The Cytokine TGF-β Promotes the Development and Homeostasis of Alveolar Macrophages

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1 Summary

Alveolar macrophages (AMs) are lung resident macrophages that reside in the lumen of small airways including alveoli. They play a critical role in lung homeostasis and maintain respiratory functions via surfactant clearance within the alveolar space. Like most other tissue-resident macrophages, AMs derive from fetal liver monocytes, which colonize the developing lung during embryogenesis and give rise to fully mature AMs perinatally. Recently, AM gene expression profile has been revealed and AM-specific transcription factor PPAR-γ has been identified. However, extracellular signaling mediators involved in AM development and maintenance have not been fully identified and the regulatory mechanisms related to AM genesis are still under investigation. It was previously shown that granulocyte macrophage colony stimulating factor (GM-CSF), an epithelial cell-derived cytokine is required for AM differentiation and *Pparg* expression, which is also critical for the development of AMs. Whether additional factors are involved in AM regulation is largely not known. Transforming growth factor-β (TGF-β), a multifunctional cytokine, is highly involved in cell fate determination and has been shown centrally involved in the genesis of Langerhans cells (LCs) and microglia activation. This indicates a possible role of TGF-β receptor (TGF-βR) signaling in regulating other myeloid cell populations. Since the role of TGF-βR signaling in the development of other macrophages is largely unknown, we investigated its function across different tissue-resident macrophages.

Here we reported that AM differentiation and maintenance, in contrast to most other tissue macrophages, are also dependent on TGF-βR signaling. Specifically, conditional deletion of *Tgfbr2*, the non-redundant receptor for initiating TGF-βR signaling events, in mice at different time points halted the development and differentiation of AMs. Meanwhile, TGF-β was also found to be critical for AM homeostasis beyond the perinatal stage, as conditional tamoxifen-induced deletion of *Tgfbr2* in adult $R26^{CreER}$ mice led to reduction of AMs in number. Further, we identified the source of TGF-β being AMs themselves, indicating an autocrine loop

that promotes AM self-maintenance. The transcriptomics analysis of *Tgfbr2*-deficient AMs perinatally revealed that TGF-β instructs AM signature and differentiation-related gene expression. Mechanistically, we proposed that in parallel with GM-CSF, TGF-βR signaling results in upregulation of PPAR-γ, contributing to the establishment of fully differentiated AM identity.

Altogether, we demonstrate that TGF-βR signaling is critical for the genesis, maturation and survival of AMs. These findings reveal an additional layer of complexity regarding the guidance cues, which govern the formation and diversity of tissue-resident macrophages.

2 Zusammenfassung

Alveolarmakrophagen (AMs) sind die Gewebs-Makrophagen der Lunge und sind im Lumen der kleinen Atemwege sowie den Lungenbläschen sessil. Sie spielen eine kritische Rolle bei der Lungenhomöostase indem sie die Lungenfunktion durch die Beseitigung von Surfactant im Lungenraum aufrechterhalten. Wie die meisten anderen gewebespezifischen Makrophagen, entwickeln sich Alveolarmakrophagen aus den Monozyten der fetalen Leber, welche während der Embryogenese die sich-entwickelnde Lunge besiedeln und zu Alveolarmakrophagen reifen. Erst kürzlich wurde PPAR-γ als spezifischer Transkriptionsfaktor für Alveolarmakrophagen identifiziert und die spezifische Genexpression der Alveolarmakrophagen aufgelöst. Jedoch ist noch nicht viel über die extrazellulären Faktoren, welche von den Alveolarmakrophagen sekretiert werden und die Regulationsmechanismen, denen die Identität der Alveolarmakrophagen unterliegt, bekannt. Die aktuelle Forschung fokussiert sich hauptsächlich auf den Granulozyten-Makrophagen-Kolonie-stimulierenden Faktor (GM-CSF), ein Zytokin, welches vom Epithel sekretiert wird und wichtig für die Entwicklung der Alveolarmakrophagen und deren *Pparg* Expression ist. Allerdings ist noch nicht bekannt, ob zusätzliche Faktoren bei der Entwicklung der Alveolarmakrophagen eine Rolle spielen. Der ‚transformierende Wachstumsfaktor beta' (TGF-β) ist für Gewebs-Makrophagen ein multifunktionelles Zytokin, welches für das Zellschicksal der Alveolarmakrophagen, die Entstehung der Langerhans-Zellen sowie die Aktivierung von Mikroglia von hoher Relevanz ist. Aufgrund dessen könnte der TGF-β Rezeptor Signalweg auch eine bedeutende Rolle für andere myeloide Zellen einnehmen.

Wir konnten zeigen, dass Alveolarmakrophagen im Gegensatz zu vielen anderen gewebespezifischen Makrophagen vom TGF-β Rezeptor Signalweg abhängig sind. Eine konditionelle Deletion des TGF-β Rezeptor, dem nicht-redundanten TGF-β Rezeptor, zu unterschiedlichen Zeitpunkten verhinderte die Entwicklung und Differenzierung der Alveolarmakrophagen in Mäusen. TGF-β ist zudem auch nach der Geburt für die Homöostase der Alveolarmakrophagen wichtig, da eine Tamoxifen-induzierte Depletion des TGF-βR2 in adulten $R26^{CreER}$ Mäusen eine Reduktion der Alveolarmakrophagen verursachte. Als Quelle des TGF-β identifizierten wir Alveolarmakrophagen selbst, was auf eine autokrine

Selbstversorgung hindeutet. Die Transkriptom-Analyse der perinatalen TGF-βR2-defizienten Alveolarmakrophagen verdeutlichte, dass TGF-β deren spezifische und Differenzierungs-bezogene Genexpression reguliert. Wir stellen die Hypothese auf, dass TGF-β ebenso wie GM-CSF die Expression von PPAR-γ, eines spezifischen Transkriptionsfaktors für Alveolarmakrophagen, reguliert.

Abschließend konnten wir zeigen, dass der TGF-β Rezeptor Signalweg für die Entwicklung, Reifung sowie das Überleben von Alveolarmakrophagen von Bedeutung ist. Diese Erkenntnisse zeigen die Komplexität, welcher die Entstehung und Diversität der gewebespezifischen Makrophagen unterliegen.

3 Abbreviations

AEC	Alveolar epithelial cell
AGM region	Aorta, gonads, and mesonephros
AIRE	Autoimmune regulator
AP-1	Activator protein 1
BM	Bone marrow
CCR2	C-C chemokine receptor type 2
cDC	Conventional dendritic cell or Classical dendritic cell
CDP	Common dendritic cell progenitor
CLP	Common lymphoid progenitors
cMoP	Common monocyte progenitor
CMP	Common myeloid progenitor
COPD	Chronic obstructive pulmonary disease
CREB	Cyclic AMP-responsive element-binding protein
CSF-1	Colony-stimulating factor 1 (also known as M-CSF)
CX3CR1	CX3 chemokine receptor 1 (also known as fractalkine receptor)
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
EMP	Erythro-myeloid precursors
FcγRI	Fc-gamma receptor 1 (also known as CD64).
FKBP12	12 kDa FK506-binding protein
Flt3L	Fms-like tyrosine kinase 3 ligand
GARP	Glycoprotein A repetitions predominant protein
GM-CSF	Granulocyte macrophage colony stimulating factor
GMP	Granulocyte-monocyte progenitor
HSC	Hematopoietic stem cell
IFN	Interferon
IL-1β	Interleukin-1 beta
IL-23	Interleukin-23

IMF	Interstitial macrophages
iNOS	Nitric oxide synthases
IRF	Interferon regulatory factor
Itgam	Integrin alpha M (also known as CD11b)
Itgax	Integrin alpha X (also known as CD11c)
iTreg	Induced Treg
JAK/STAT	Janus kinase-signal transducer of activation of transcription
LAP	Latency-associated peptide
LC	Langerhans cell
LPS	Lipopolysaccharides
LTBP	Latent TGF-β binding protein
Ly6C	Lymphocyte antigen 6 complex, locus C1
MAPK	Mitogen-activated protein kinase
MdC	Monocyte-derived cell
MDP	Macrophage dendritic cell progenitor
MEP	Megakaryocyte-erythrocyte progenitor
MerTK	Proto-oncogene tyrosine-protein kinase MER
MHCI	Major histocompatibility complex class I
MHCII	Major histocompatibility complex class II
moDC	Monocyte-derived dendritic cell
MPS	Mononuclear phagocyte system
NF-κB	Nuclear factor-κB
NK cells	Natural killer cells
NLR	NOD-like receptors
nTreg	Natural Treg
PAMP	Pathogen-associated molecular pattern
PAP	Pulmonary alveolar proteinosis
PAR6	Partitioning defective 6
pDC	Plasmacytoid dendritic cell
PRR	Pattern recognition receptor

PU.1	Transcription factor PU.1
RAG	Recombinase-activating gene
RLR	RIG-I-like receptors
Siglec-F	Sialic acid-binding Ig-like lectin F
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
TGF-β	Transforming growth factor beta
TGF-βR	TGF-β receptor
TLR	Toll-like receptors
TNFα	Necrosis factor alpha
Treg	Regulatory T cell

4 Introduction

4.1 The Immune System

The immune system is a host defense system against a full range of invading foreign agents including particles, viruses, bacteria and parasites, as well as diseased cells or tissue debris. Central processes in the immune response involve the recognition and clearance of non-self invading agents and abnormal self components. Dysregulation of the immune system is related to various types of human diseases. For example, a compromised or inactive immune system leads to immunodeficiency that is typically associated with recurring infections throughout life. The inability to distinguish non-self and self might result in cancer or autoimmune diseases. In addition, the immune system also contributes to the proper development and normal homeostasis of host tissue[1].

The immune system can be divided into two parts: the innate immune system and the adaptive immune system. The innate immune system performs fast first-aid immune responses, while the adaptive immune system generates responses with a time lag but with memory and high specificity. From an evolutionary standpoint, innate immunity exists in all forms of life but only vertebrates have developed a complete adaptive immune system[2]. In mammals, both innate and adaptive immune systems are composed of cells, cytokines and humoral factors. They work in a coordinated and compensatory fashion to keep health and prevent infection.

4.1.1 The Innate Immune System

The innate immune system comprises three different parts throughout the human body: the anatomic barriers, the humoral compartment of innate immunity and innate immune cells. The anatomic barriers include epithelial cell-sealed surfaces, such as the skin, the gastrointestinal tract and the respiratory tract. These barriers prevent direct pathogen entry with intercellular tight junctions. They produce mucus and harbor microbiota to counteract the colonization of pathogens. Taking the lung as an example, the beating of cilia moves mucus towards the outside opening of the respiratory tract, trapping and flushing foreign particles away. Lung epithelial cells

secrete pulmonary surfactants and ‘antibiotics’ such as α-defensins and cathelicidin which further prevent invasions[3].

In humoral innate immunity, the complement system plays a major role. It is composed of more than 30 surface and plasma proteins that follow organized proteolytic cascades after recognition of pathogen-associated molecular patterns (PAMPs), pathogenic surfaces or antigen bound antibodies. Activated through three different pathways, the complement system converges into the assembly of C3 and C5 convertase and generates core effectors: proinflammatory mediator anaphylatoxins (C4a/C3a/C5a), opsonins for opsonization of the surface of pathogens and the membrane attack complex for lysis of targeted surfaces.[4]

The most dominant players in the innate immune system are the innate leukocytes: the myeloid cells including mononuclear phagocytes (monocytes, monocyte derived cells, dendritic cells (DCs) and macrophages), granulocytes (basophils, eosinophils, neutrophils and mast cells), and a few cell populations from the lymphoid lineage such as natural killer (NK) cells, innate lymphoid cells, and γδ T cells. Phagocytosis by monocytes, macrophages, neutrophils, DCs and mast cells of various invaders such as bacteria and parasites includes internalization, either for degradation in phagolysosomes or for antigen processing by antigen presenting cells, for example DCs, for initiating adaptive immune responses. Another immune mechanism to combat infections is to directly target infected cells for killing. NK cells induce cytotoxicity of target cells via secreting perforin and granzymes, activating Fas-mediated caspase-dependent apoptosis or acting through antibody dependent cellular cytotoxicity. The killing activity of NK cells is regulated through the balance between inhibitory signals and activating signals. For example, viral infected host cells or tumor cells usually downregulate expression of the major histocompatibility complex class I (MHCI) molecule, which diminishes the inhibitory signal in NK cells and tips the balance towards the activating signals for killing.[5] During the whole processes, innate leukocytes are activated to produce chemokines and cytokines that attract different players in the innate and adaptive immune compartment, which work cooperatively.

The innate immune cells distinguish pathogens and abnormal fragments from healthy components through the recognition of conserved PAMPs from infectious microbes

or damage-associated molecular patterns (DAMPs) from host derived abnormal cellular events. Such pattern-based recognition is conducted by pattern recognition receptors (PRRs) present in different cell types, mainly Toll-like receptors (TLRs), C-type lectin receptors, NOD-like receptors (NLR), RIG-I-like receptors (RLR) and DNA sensors [6]. The patterns they can recognize cover a vast majority of pathogens, including lipopolysaccharides (LPS) from gram-negative bacteria, ssRNA, dsRNA and CpG rich DNA material from bacterial and virus, Flagellin from bacteria, β-glucans from fungus, lipoteichoic acids from gram-positive bacteria and many bacteria specific lipomannans and lipoproteins. In general, TLRs are majorly responsible for recognizing PAMPs and DAMPs present in extracellular spaces or those that have been taken into the endosome, while NLRs detect mainly pathogenic material in the cytoplasm and RLRs sense cytoplasmic viral RNAs. Upon ligand binding, PPRs initiate intracellular signaling transduction and activate primarily the transcription factors nuclear factor-κB (NF-κB), interferon regulatory factor (IRF), activator protein 1(AP-1), and cyclic AMP-responsive element-binding protein (CREB) to induce the expression of inflammatory cytokines and type I interferons (IFN).[7]

4.1.2 The Adaptive Immune System

Usually, the activation of innate immunity is followed by adaptive immune system-mediated responses that target antigens specifically and further keep the memory for the same antigen upon a second challenge. The two key players in adaptive immune system are T lymphocytes and B lymphocytes, which both make use of a vast diversity of antigen specific receptors. The diversity in T cell receptor (TCR) and B cell receptor (BCR) pool is generated via somatic DNA rearrangement and random chain pairing during cell development. Once they meet an antigen, the corresponding antigen specific B or T cells become activated and undergo clonal expansion, becoming numerous effector T cells for cytokine production, immune regulation or cytotoxicity induction or plasma cells for antibody production.

T cells develop from common lymphoid progenitors (CLP) in the bone marrow (BM), which later migrate to the thymus where they mature into naive T cells. In the thymus the gene regions encoding TCR chains undergo somatic rearrangement of variable (V), diversity (D) and joining (J) fragments to generate mature VJ α-chains and VDJ

β-chains. This process is dependent on the lymphoid-specific recombinase-activating gene (RAG) 1 and RAG2 proteins for DNA elements cleavage and terminal deoxynucleotidyl transferase (TdT) for extra VDJ junction modification and diversity generation[8]. This generates a huge variety of T cell clones that later become $CD4^+CD8^+$ double positive. The double positive immature T cells are first positively selected in the thymic cortex for proper affinity of the TCR to self-major histocompatibility complex (MHC) class II (MHCII) molecules. During the positive selection, they become either $CD4^+$ single positive if they are selected on MHCII molecules of cortical thymic epithelial cells or $CD8^+$ single positive if they are selected on MHC class I (MHCI) molecules. The single positive immature T cells move to thymic medulla for negative selection so that self-reactive T cell clones are removed from the T cell pool under the control of a gene called autoimmune regulator (AIRE), expressed in medullary thymic epithelial cells.[9] After positive and negative selection, naive mature T cells leave the thymus and migrate to secondary lymphoid tissues. When a naive $CD4^+$ T cell encounters a cognate antigen presented on an MHCII molecule on the surface of antigen presenting cells or when a naive $CD8^+$ T cell recognizes its corresponding antigen presented by MHCI molecules, naive T cells are activated and undergo clonal expansion and differentiate into effector T cells. Effector T cells, that is $CD4^+$ T helper (Th) cells and $CD8^+$ cytotoxic T cells, migrate in the periphery to the site of infection. T helper cells mainly secrete cytokines to boost and control the overall immune response as well as assist B cell activation. Cytotoxic T cells directly target cells bearing a specific antigen by MHCI molecules for killing. Effector T cells can remain, after pathogen clearance, as effector memory T cells in the periphery, and in the lymph node activated T cells can also stay as central memory T cells. $CD4^+$ T cells can also develop into $CD25^+$ $Foxp3^+$ regulatory T cells (Tregs) either in the thymus as natural Tregs (nTregs), or in the periphery as induced Tregs (iTregs). They play a central role in modulating immune responses and regulating peripheral tolerance.[10]

B cells develop in fetal liver and adult BM from CLPs. Similar to T cells, the maturation of B cells involves somatic rearrangement-mediated BCR repertoire formation. During antigen-dependent activation in peripheral lymphoid tissues, naive B cells become plasma cells after recognition of polymeric antigen or under the assistance of Th cells. The latter involves isotype switching, which generates BCRs

with the same antigen specificity but different effector functions and induces somatic mutation which leads to affinity maturation[8]. Plasma cells are professional antibody producing cells, releasing the secreted form of BCRs. Like T cells, the activated B cells can develop into memory cells for maintaining long-lived plasma cells, which constantly secrete antibodies and keep humoral immune surveillance[11].

4.2 The Ontogeny and Function of Monocytes, Dendritic Cells and Tissue-Resident Macrophages

Monocytes, macrophages and DCs were classified together as the mononuclear phagocyte system (MPS) because they have previously been thought to share great similarity in morphology, ontogeny and function[12]. However, accumulating evidence shows that despite being mononuclear phagocytic cells, they have distinct functions in the immune system. Moreover, recent findings have overturned the old concept that BM-derived monocytes give rise to the rest of cell populations in the MPS. In fact, under non-inflammatory conditions, DCs and monocytes originate from two parallel branches, both of which are derived from macrophage dendritic cell progenitors (MDPs) in the BM. Under physiological condition, most macrophage populations are embryonically derived. Inflammation induced monocyte-derived cells (MdCs), typically referred to as monocyte-derived macrophages or monocyte-derived dendritic cells (moDCs), are phenotypically and functionally different from the conventional/classical (c)DCs or tissue-resident macrophages. (Figure.1.)

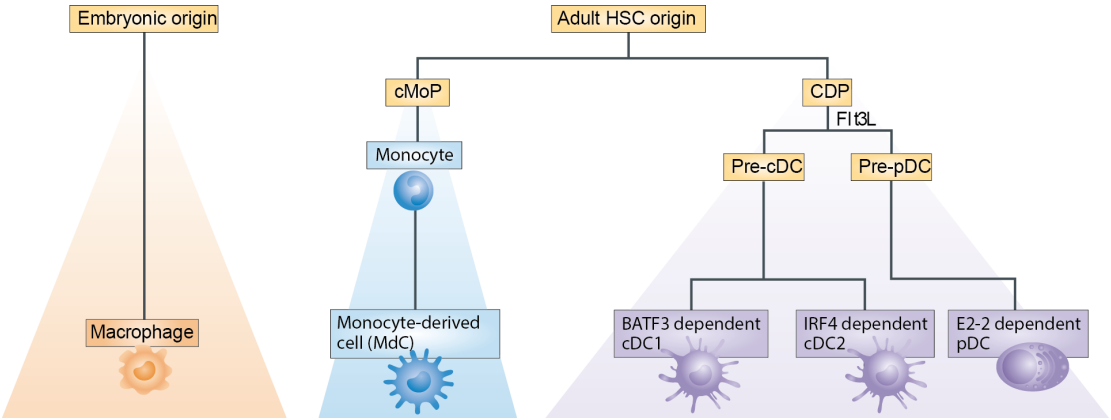

Figure 1. Classification and ontogeny of mononuclear phagocytes. Mononuclear phagocytes are classified based on their ontogeny into three main groups: embryonic-derived macrophages, monocytes/monocyte-derived cells (MdCs), and common dendritic cell precursor (CDP)-derived dendritic cells (DCs). Dependent on different sets of transcription factors during development, DCs are further divided into classical type 1 DCs (cDC1s), classical type 2 DCs (cDC2s) and plasmacytoid DCs (pDCs). Adapted from reference[13]

4.2.1 Dendritic Cells

DCs are sub-classified into cDCs and plasmacytoid dendritic cells (pDCs). pDCs are a rather separate population. They are specialized type I IFN producers after viral nucleic acids recognition and they can be easily distinguished from the rest of cells in

the MPS by B220 and Siglec-H expression[14]. Langerhans cells (LCs), the epidermal resident myeloid population, are often excluded when we mention cDCs because they are derived from embryonic precursors similar to tissue-resident macrophages and are therefore ontogenetically unrelated to cDCs [15,16]. Experimentally, cDCs can be identified by the surface expression of MHCII and the integrin alpha X (Itgax, also known as CD11c), and the lack of macrophage markers such as proto-oncogene tyrosine-protein kinase MER (MerTK) and Fc-gamma receptor 1 (FcγRI, also known as CD64).

cDCs are professional antigen presenting cells in the immune system. Like macrophages, they express PPRs and scavenger receptors to sense and take up non-self particles, but they make use of the phagocytosis pathway to achieve their superior capability of antigen processing and presentation to naive T cells. By doing so, cDCs prime and reinforce T cell-mediated adaptive immune response to foreign antigens. On the other hand, they play a crucial role in inducing tolerance via the suppression of T cell responses and the control of regulatory T cell genesis and function[17].

cDCs are short-lived and constantly repopulated by BM-derived precursors. In the BM, all myeloid cells (DCs, monocytes, and granulocytes) are developed from common myeloid progenitors (CMP) which further give rise to megakaryocyte-erythrocyte progenitors (MEPs) or granulocyte-monocyte progenitors (GMP)[18]. GMPs later develop into MDPs, which lose the potential to generate granulocytes and are committed to the DC and monocyte lineages only[19,20]. From MDPs, two lineages diverge: common monocyte progenitors (cMoP), which are unipotent in differentiating into monocytes, and common dendritic cell progenitors (CDP), which represent the first dedicated DC progenitor stage[21]. Depending on the presence of Fms-like tyrosine kinase 3 ligand (Flt3L) and the surface receptor Flt3 expression, CDPs are able to give rise to both pDCs and pre-DCs[22]. Pre-DCs leave the BM and seed all the tissues, which later fully differentiate into two cDC lineages: the cDC1 and cDC2. Some evidence also indicates that the commitment to two cDC lineages happens at the CDP stage[23].

Typically, cDC1 refers to $CD103^+$ cDC in nonlymphoid tissues and $CD8^+$ cDCs in lymphoid tissues, while cDC2 refers to $CD11b^+$ (also known as integrin alpha M, Itgam) cDC in both lymphoid and nonlyphoid tissues[14,24]. Experimentally, both

cDC1s and cDC2s are positive for MHCII and CD11c within the $CD45^+$ compartment. The migratory DCs from non-lymphoid tissues to lymphoid tissues can be distinguished by their elevated MHCII expression and low expression of CD11c. Phenotypically, in many tissues cDC2s often express macrophage markers, such as CD172a, F4/80, and CX3CR1, that cDC1s lack. Most of the cDC1s are positive for CD24, CD26, Clec9a, langerin and the chemokine receptor XCR1, and have higher level of Flt3 expression than cDC2s[14,24,25]. More stringently, the cDC1 and cDC2 subsets are determined based on the transcriptional regulators required for their development. cDC1s are dependent on transcriptional factor Batf3 and IRF8 and the inhibitor of DNA protein Id2 in their developmental processes[26–29]. On the contrary, the more heterogeneous cell population of cDC2s are independent of Batf3, IRF8 and Id2 but require IRF4, Zeb2, RelB and other different transcriptional factors for their development in different tissues[14,30–33]. Functionally, cDC1s present antigens preferably to CD8+ T cells via cross-presentation on MHCI and cDC2s have better capability in antigen presentation via MHCII[14]. cDC1s are found to be important in directing the differentiation of CD4+ T cells towards a Th1 phenotype via IL-12 secretion and iTregs induction[34]. cDC2s promote Th2 responses as well as being the main IL-23 producers in mediating Th17 responses[35–38]. The same subsets of cDCs are present across different tissues. It is becoming evident that the distinct cDC1 or cDC2 ontogeny rather than their specific tissue microenvironment dictates the major differences that exist in these two cDC populations.

4.2.2 Monocyte and Monocyte Derived Cells

Monocytes are a short-lived cells and are constantly replenished by BM cMoPs independent of Flt3 [21,39,40]. In the blood stream, two populations exist within the monocyte compartment: $Ly6C^{hi}$ monocytes, which express high levels of lymphocyte antigen 6 complex, locus C1 (Ly6C) and $Ly6C^{lo}$ monocytes. Both populations express the colony-stimulating factor 1 (CSF-1; also known as macrophage colony-stimulating factor, M-CSF) receptor (CSF-1R, M-CSFR), CD11b, and F4/80 positive. $Ly6C^{hi}$ monocytes, also referred to as classical monocytes, express additionally L-selectin, CD62L, C-C chemokine receptor type 2 (CCR2), and intermediate levels of CX3 chemokine receptor 1 (CX3CR1, fractalkine receptor)[41]. $Ly6C^{lo}$ monocytes, known as patrolling monocytes, express low levels of Ly6C but high levels of

CX3CR1 and leukosialin (CD43). They lack CCR2 and CD62L expression. Ly6C^{hi} monocytes are directly differentiated from cMoPs and exit from BM in a CCR2 dependent manner[42]. In the blood, Ly6C^{hi} monocytes give rise to Ly6C^{lo} monocytes in a CSF-1R and CX3CR1/Nur77 dependent manner[40,43,44].

Functionally, Ly6C^{lo} monocytes have been less studied. They are believed to be the counterpart of a 'tissue-resident macrophage' population in the blood, because of their prominent role in endothelial tissue homeostasis. It has been shown that Ly6C^{lo} monocytes survey vessel integrity via phagocytosis of cellular debris and initiate tissue repair[45,46]. During inflammation and upon pathogenic infection, Ly6C^{hi} monocytes enter tissues and differentiate into effector cells termed as MdCs, which are usually Ly6C^{hi} but resemble cDCs or tissue-resident macrophage in expressing CD64, MHCII, and CD11c[41]. The mechanism in deciding the differentiation into moDCs or monocyte-derived macrophages is still under debate[47]. MdCs are quite heterogeneous populations in individual cases and sites of infection or inflammation. They are capable of synthesizing nitric oxide synthases (iNOS), and secreting cytokines such as tumour necrosis factor alpha (TNFα), Interleukin-1 beta (IL-1β), Interleukin-23 (IL-23) and IFN-γ[48–50]. MdCs can also acquire antigen-presenting properties and thus are able to prime T cells[41,48]. Tissue infiltrating Ly6C^{hi} monocytes and MdCs are the main effector cells responsible for tissue damage during inflammation. For example during experimental autoimmune encephalomyelitis (EAE), disease progression correlates with the number of infiltrating moDCs[51,52]. In summary, monocytes and MdCs are important participants during inflammation and the pathogenesis of different infections and autoimmune diseases.

4.2.3 Tissue-Resident Macrophages

Macrophages are the resident populations present in all organs of the body. In general, unlike DCs and monocytes that are mainly involved in inflammation and adaptive immune responses, tissue-resident macrophages are specialized phagocytic cells dedicated in maintaining tissue homeostasis and integrity. Depending on which tissue they reside in, tissue-resident macrophages exhibit great heterogeneity in terms of development, maintenance and function[24,53]. Experimentally, most tissue-resident macrophages can be identified by the surface expression of CD11b, F4/80, CSF-1R,

MerTK and CD64 and a lack of Ly6C, though diversity of surface marker expressions exist among different tissue-resident populations[24,53].

As mentioned previously, the long-held dogma that BM-derived circulating monocytes migrate into peripheral tissues and differentiate into issue-resident macrophages has been largely revised. The majority of adult tissue-resident macrophage populations have embryonic precursors which seed the tissue before birth[15,54]. During embryogenesis, the hematopoietic system is gradually established in three waves (Figure 2). First, around embryonic day 7 (E7) early progenitors emerge from the blood islands of the extra-embryonic yolk sac, giving rise to primitive macrophages, erythroblasts and megakaryocytes. This stage is termed primitive hematopoiesis. The second wave, the transient definitive wave of erythro-myeloid precursors (EMPs), arise from the yolk sac hemogenic endothelium between E8.0 and E8.5[16]. This 'late' EMPs have the potential to give rise to erythroid and myeloid cells, but not to lymphoid lineages. After the blood circulation is established from E9.5, EMPs migrate into the fetal liver, where they give rise to different lineages, including fetal liver monocytes. Finally, definitive hematopoiesis starts from the intraembryonic hemogenic endothelium at E10.5. The immature hematopoietic stem cells (HSCs) first arise in the para-aortic splanchnopleura region and give rise to fetal HSCs in the aorta, gonads, and mesonephros (AGM) regions[55,56]. These precursors colonize both fetal liver and BM for the establishment of definitive hematopoiesis. From E12.5, the fetal liver becomes the major hematopoietic organ with precursors from both transient definitive and definitive hematopoietic waves[55,56].

The three waves of progenitors contribute differently to various tissue-resident macrophages along different time points in embryogenesis and adulthood. Yolk sac primitive macrophages are the only source of microglia throughout life since their residence in the brain from E9.5[57]. In other organs, after primitive macrophages seed the tissues they remain dominant only between E10 and E13. Afterwards they are gradually replaced by fetal liver monocyte-derived macrophages, which are proposed to be originated from EMPs and with limited HSC involvement[15,16]. Despite this, circulating monocytes contribute to a various extent to different tissue-resident macrophages under normal homeostasis after birth. Microglia in the brain, LCs in the epidermis, alveolar macrophages in the lung, and splenic red pulp macrophages, self-

renew locally and remain of embryonic origin without monocyte contribution[55,57–60]. Monocytes can differentiate into arterial macrophages and Kupffer cells in the liver within a short period after birth and, together with embryonic derived macrophages, self-maintain locally[61,62]. In contrast, macrophages in the gut, dermis, heart and pancreas are not capable of self-maintenance and are thus constantly repopulated by monocytes in adult[63–67]. Besides that, under certain conditions such as with severe pathological inflammation or upon lethal irradiation in a BM transplant setting, monocytes come to fill up the empty niche of tissue-resident macrophages.

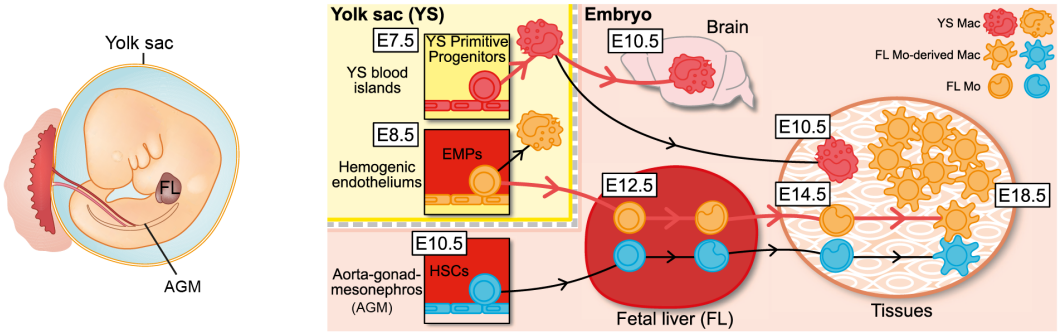

Figure 2. The three waves of embryonic hematopoiesis and their contribution to tissue-resident macrophages. Left**:** Illustration of yolk sac (YS), fetal liver (FL) and aorta-gonads-mesonephros (AGM) region in the mouse embryo. Right: Hematopoiesis emerges in three sequential programs. YS primitive progenitors give rise to the first wave of YS macrophages (red) at E7.5, which dominate in all tissues at E10.5 but only persist in the brain as microglia. Erythro-myeloid precursors (EMPs) that are generated at E8.5 in the hemogenic endothelium of the yolk sac initiate the second wave of hematopoiesis (orange). They give rise to FL monocyte which later seed every tissue with the exception of brain and differentiate into most adult resident macrophage populations. The third wave (blue) starts at E10.5 with the emergence of the hematopoietic stem cells (HSC) from the embryo proper hemogenic endothelium of the aorta-gonads-mesonephros (AGM) region. They can also generate fetal liver monocytes with limited contribution to tissue resident macrophages. Adapted from references[16,68]

In general, by expressing Fc-receptors, complement receptors, PRRs, scavenger receptors and cytokine receptors, tissue-resident macrophages are capable of recognition, phagocytosis and degradation of pathogens and tissue debris, as well as sensing different stimuli and inducing immune responses and tissue repair[69]. In detail, despite having a similar developmental origin, tissue-resident macrophages mature into functionally different populations[70] depending on diverse microenvironments and functional requirements of different organs. For example, adipose-associated macrophages mainly control insulin sensitivity and adaptive thermogenesis[71,72]. Alveolar macrophages are specialized in lung homeostasis and surfactant catabolism[73]. Microglia are highly involved in neuronal homeostasis and synaptic

remodeling[74,75]. Splenic red pulp macrophages and Kupffer cells support iron homeostasis as well as the clearance of blood carrying aged cells and debris[76,77]. Bone marrow macrophages play an important role in erythropoiesis and attaching hematopoietic stem cells to stem cell niches[78–80].

Common extrinsic and intrinsic factors critical for macrophage development and homeostasis have been identified. The development and survival of macrophages is largely dependent on CSF-1. Mice lacking CSF-1 show a severe reduction of most tissue-resident macrophage numbers[81]. The generation of primitive macrophages and hence microglia is a c-Myb expression-independent process, while subsequent hematopoietic waves require c-Myb. All primitive macrophages and adult tissue-resident macrophages require transcription factor PU.1 (PU.1, encoded by *Spi1*), a pioneer transcription factor for their development and maintenance[82,83]. PU.1 is capable of binding to closed chromatin prior to macrophage lineage commitment and creating accessibility for additional *trans*-regulators[84]. These include lineage-defining transcription factors and stimuli-induced transcription factors, which together dictate the differentiation into the macrophage lineage and later the heterogeneity of macrophages in different tissue microenvironment, respectively[85,86]. The transcription factors c-Maf and MafB are key negative regulators of macrophage self-renewal[87]. A low expression of MafB and c-Maf in macrophages ensures the induction of genes including *Myc, Klf2* and *Klf4* with accessible enhancers and subsequently the self-renewal capacity[88].

In recent years, unique gene expression profiles, specific transcriptional factors together with distinct sets of enhancers in each tissue macrophage population have been largely revealed[53,85,89]. These cell intrinsic factors include Sall1 for microglia, Peroxisome proliferator-activated receptor gamma (PPAR-γ) for alveolar macrophages, Gata6 for peritoneal macrophages, and Spi-C for splenic red pulp macrophages, which define the identity of each tissue-resident macrophage population.

However, ontogeny alone could not fully explain why macrophages of similar origins have differential transcription factor gene expression. Evidence further indicates that the tissue microenvironment is non-redundant in shaping the identity of macrophages. Primitive macrophages, fetal liver monocytes, and adult monocytes can all develop

into functional 'alveolar macrophages' when they are transferred into the alveoli[90]. Terminally differentiated peritoneal macrophages can be reprogrammed by the lung microenvironment to exhibit alveolar macrophage-like phenotypes[85]. Altogether, identifying tissue related soluble factors and the underlying regulatory pathways for different macrophage populations become increasingly important for understanding macrophage development. For example, stromal cell-derived IL-34 is required by microglia for their maintenance and by LCs for their development and maintenance[91]. LCs are also dependent on TGF-β for inducing Id2 and Runx3 during their differentiation and homeostasis[92,93]. AMs on the other hand require granulocyte macrophage colony stimulating factor (GM-CSF, encoded by *Csf-2*) to maintain PPAR-γ expression for their development, survival and function[60,94]. Heme has been shown to induce Spi-c for red pulp macrophage development[77,95]. Retinoic acid elevates Gata6 expression and therefore contributes to the development of peritoneal macrophages[96]. Whether there are other tissue specific factors and how the overall regulatory network works remains elusive.

4.3 Alveolar Macrophages

AMs are resident macrophages in the lung, which reside in the lumen of bronchi, bronchioles, and alveoli (Figure 3). Current studies did not distinguish macrophages that are exactly located in alveoli from those that are found in larger airways. AMs differ from a second macrophage population in the lung, the interstitial macrophages (IMF) that are located within the lung parenchyma. Phenotypically, AMs express common macrophage surface molecules like CD64, MerTK, and F4/80 and are negative for MHCII. In addition, they express high levels of CD11c and sialic acid-binding Ig-like lectin F (Siglec-F) and low levels of CD11b, which distinguishes them from the Siglec-F^{-}CD11b^{+} IMF[60]. Compared to other tissue-resident macrophages, AMs are kept in an immune suppressive state under homeostatic conditions and even during inflammation, they coordinate defense against infection and protection against tissue damage[73,97,98]. Loosely attached to alveolar epithelial cells (AECs), AMs closely interact with lung epithelium and alveolus microenvironment and thus exist as an indispensable cell type for pulmonary tissue homeostasis.

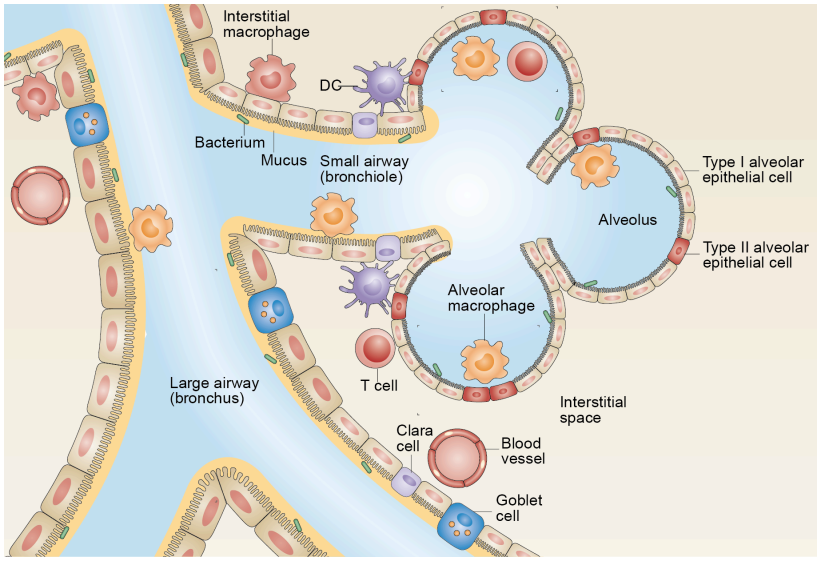

Figure 3. Overview of leukocytes and terminal airway structures in the healthy lung. Alveolar macrophages (AMs) reside in the airspaces next to either type I alveolar epithelial cells (AECIs) which are the sites of gas exchange and account for a majority of the total surface area of the lungs or next to type II alveolar epithelial cells (AECIIs) which are responsible for surfactant secretion. Inside the lumen of the healthy lung, AMs are the only dominant cell population, though eosinophils (not shown) and T cells can exist. Macrophages found in the larger airways (bronchi and bronchioles) and typically located within the mucous layer are also grouped into AMs. Beside AECs, lung epithelia also contain mucus-producing goblet cells and secretory non-ciliated Clara cells. In the interstitial space between the alveoli and the blood vessels, interstitial macrophages, T cells, dendritic cells and a sparse population of B cells (not shown) also reside. Commensal (and pathogenic) bacteria reside within the airway mucosa and in the alveoli. Adapted from reference[73]

4.3.1 The Function of Alveolar Macrophages

Like other macrophages, AMs have the phagocytic ability to clear apoptotic cells, airborne agents, fungi and bacteria and have been shown to promote the defense against influenza virus infection[94]. Constantly exposed to the outside environment with excessive airborne particles, AMs have unique cell properties to prevent unnecessary inflammatory responses, resolve inflammation and avoid uncontrolled tissue injury. Early studies showed that lung macrophages secrete the cytokine TGF-β and suppress pulmonary immune responses by downregulating antigen-presentating functions of DCs[99,100]. It has been well established that depending on TGF-β and retinoic acids, AMs promote the generation of iTregs and hence promote airway tolerance towards innocuous inhaled antigens[101]. The immune suppressive function of AMs is also mediated by inhibitory receptors on AMs that bind to the ligands presented by AECs or in the alveolar fluid. The interaction between CD200L on AECs and CD200R on AMs and the recognition of surfactant proteins SP-A and SP-D by signal-regulatory protein SIRPα on AMs, negatively regulates TLR-induced pro-inflammatory responses[73]. Only combined PRR ligation that overrides the inhibitory signals will successfully activate AMs. For protecting tissues from injury and resolving inflammation, AMs upregulate Trem2 and mannose receptor (CD206) expression with a strengthened IL-10R ligation via an increased IL-10 supply from the extracellular space. These receptors on AMs cooperatively help to balance excessive IFN-γ, IL-1, TNF-α, and PRR downstream signaling[73]. Clinically, activated AMs or disappearance of resident AMs are often associated with pulmonary disorders such as allergic asthma and chronic obstructive pulmonary disease (COPD)[102–104].

Through removing inhaled particles, pathogens, matrix materials, apoptotic cells and tissue debris in an inflammation suppressive manner, AMs maintain the lung tissue integrity. Among them, one major role of AMs is the uptake and catabolism of surfactant, extracellular proteins and lipids that are secreted by type II AECs, which helps to lower the surface tension of the lung epithelium. Failure of AMs to do so leads to surfactant accumulation in the alveolar space and impairment of the respiratory function of the lung. In humans, this is termed pulmonary alveolar proteinosis (PAP) and has been recapitulated in *Csf2*$^{-/-}$ or *Csf2rb*$^{-/-}$ mice, which lack GM-CSF or its receptor and are hence devoid of AMs[105,106]. Dysfunctional AMs have

also been implicated in PAP independent emphysema. Mice lacking αvβ6, an integrin that is expressed on the surface of respiratory epithelial cells and mediates latent TGF-β activation, will develop emphysema and lung inflammation[107].

4.3.2 The Developmental Pathway of Alveolar Macrophages

AMs develop from fetal liver monocytes and these embryonically-derived AMs persist in the tissues throughout life in the steady state without the contribution of BM-derived precursors[41,58]. Briefly as previously described, fetal monocytes develop from late EMPs generated in the yolk sac at E8.5 and emerge in the fetal liver from E12.5[15,16]. They begin to accumulate in the developing lung at E14.5 and then differentiate into immature AMs (preAMs), which mature into AMs postnatally[60]. During this process, fetal liver monocytes which are Ly6C^{hi}, CD11b^{hi} and F4/80^{+} down regulate Ly6C and CD11b when they differentiation into preAMs in the lung. These preAMs remain negative for Siglec-F and CD11c expression and only become fully mature after postnatal day (P)3 with high levels of Siglec-F and CD11c expression[60]. (Figure 4)

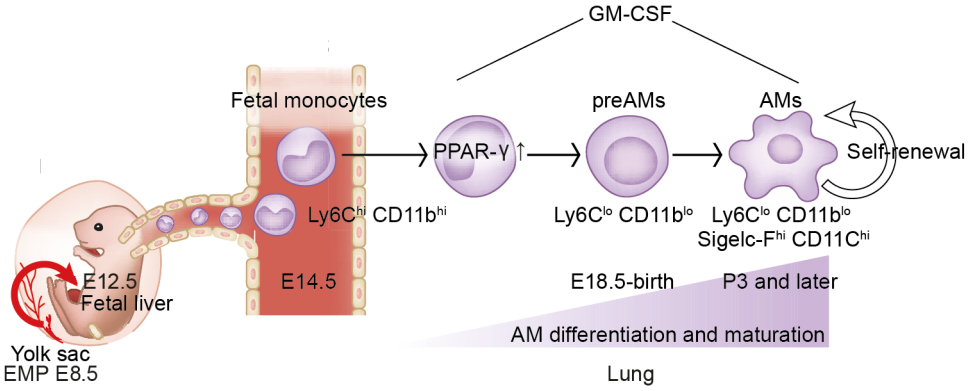

Figure 4. The developmental pathway of AMs. AMs are fetal liver monocyte-derived and self-maintain locally after birth. Fetal liver monocytes are generated around E12.5 from EMPs that emerge in the yolk sac at E8.5. They start to migrate to the developing lung at E14.5 and then differentiate into immature AMs (preAMs) at E18.5, which mature into AMs postnatally. During this course, fetal liver monocytes (Ly6C^{hi} CD11b^{hi}) down regulate Ly6C and CD11b when they differentiate into preAMs in the lung. These preAMs remain negative for Siglec-F and CD11c expression and only become fully mature postnatally with high levels of Siglec-F and CD11c expression. Afterwards, AMs self-renew locally throughout life. Induction of the AM-differentiation is dependent on GM-CSF. Ligation of GM-CSFRs induces PPAR-γ expression in pulmonary fetal monocytes and instructs AM identity. Adapted from reference[108].

The genesis of AMs is highly dependent on GM-CSFR signaling as both *Csf2*$^{-/-}$ *and* *Csf2rb*$^{-/-}$ mice are devoid of AMs[105,106]. GM-CSFR signaling has been shown to be

essential for the differentiation of fetal monocytes into immature AMs perinatally and for the full maturation of AMs postnatally but not for the accumulation of fetal monocytes in the developing lung[60,94]. GM-CSF has been previously considered as a DC and pro-inflammatory cytokine as it is involved in the maintenance of homeostasis of cDC1 and some cDC2 populations in non-lymphoid tissues, and the genesis of moDCs in inflammatory settings[109–112]. How GM-CSF influences macrophages, in particular AMs, is now under investigation. In general, GM-CSF triggers a wide range of downstream signaling events including activation of the janus kinase-signal transducer of activation of transcription (JAK2/STAT5) pathway as well as activating the SRC family kinases, the PI3K pathways and the mitogen-activated protein kinase (MAPK) cascade[113]. It has been shown that GM-CSF-mediated AM differentiation and homeostasis are largely dependent on the macrophage pioneer transcription factor PU.1[114]. Equally important, GM-CSF influences the induction of the transcription factor PPAR-γ, a key factor for the transcriptional program specific to AMs[94] (Figure 4). Independently of GM-CSF signaling, the transcription repressor Bach2 is critical in AM function and surfactant homeostasis of the lung[115], indicating a more complicated regulatory mechanism than the current 'one cytokine for one key transcription factor' model. Whether during the development of AMs other cytokines are also involved is largely unknown and worth to be investigated.

4.4 The TGF-βR Signaling

The transforming growth factor beta (TGF-β) receptor (TGF-βR) signaling pathway is one of the most ancient and prevailing pathways found in metazoans. The emergence of orthologues of its core players dates back to the earliest animal species, *Trichoplax adhaerens* ('flat animals', a free-living organism with the simplest structure in all multicellular animals)[116]. It has fundamental and diverse functions in regulating the survival, pluripotency, growth, division and differentiation of embryonic stem cells, lineage progenitors and differentiated cells[117]. At tissue and organ levels it controls morphogenesis, homeostasis and regeneration in both dose-dependent and context-dependent manner[117].

In general, the TGF-βR signaling pathway is initiated with the binding of TGF-β to two Type II receptor TGF-βR2 molecules at the surface of the cell membrane, which induces the formation of a hetero-tetrameric complex between one TGF-βR2 homodimer and one homodimer of the Type I receptor TGF-βR1. Both TGF-βR1 and TGF-βR2 are receptor serine/threonine kinases. In the complex, TGF-βR2 phosphorylates TGF-βR1, which activates the kinase activity of TGF-βR1 with the dissociation of the 12 kDa FK506-binding protein (FKBP12). The activated TGF-βR1 can bind and phosphorylate Smad2 or Smad3 downstream. Phosphorylated Smad2/3 bind to Smad4 and, as a complex, they translocate to the nucleus. Cooperating with different partner transcription factors, the Smad complex is directed to the promoter region of different sets of genes, regulating their transcriptional activities[118].

4.4.1 The Complexity in TGF-βR Signaling

The TGF-β-TGF-βR1/2-Smad axis mainly regulates cell differentiation and inhibits cell proliferation in embryonic development and tissue homeostasis. Beyond the conventional TGF-β pathway, regulations and bifurcations occur at multiple levels in its signaling transduction. This generates large complexity in signaling and phenotypic outcomes after receptor-ligand binding.

4.4.1.1 The extracellular activation of the TGF-β cytokine

The active TGF-β cytokine is in the form of a 25-kd disulfide-linked dimer. Interestingly, in the extracellular space the TGF-β dimers usually remain inactive and

stored in large latent forms (Figure 5). TGF-β is synthesized as a much larger precursor peptide that is composed of the small C-terminal mature TGF-β fragment, a rather large N-terminal fragment namely the latency-associated peptide (LAP), and a signal peptide later being removed after directing precursor peptide into endoplasmic reticulum. In the lumen of endoplasmic reticulum, TGF-β precursors are assembled into a dimer. Either before or after secretion, the LAP and the mature TGF-β cytokine part require being cleaved from each other by the endoprotease furin for activation. After cleavage, the TGF-β part remains non-covalently attached and folded into the LAP region and thus being prevented from interacting with its receptors after secretion. The whole complex is defined as the small latent complex. The LAP region facilitates the interaction and binding between the small latent complex and integrins. In most cell types, the LAP region is associated with the latent TGF-β binding protein (LTBP), together forming the large latent complex with the TGF-β cytokine. LTBP mediates the interaction and attachment of the large latent complex with extracellular matrix.[119]

To release the mature TGF-β dimer from the latent complex, a large variety of potential mediators locate in the cell surface and extracellular space. These include extracellular matrix protein, *e.g.* Thrombospondin 1, for dissociating the latent complex[120–122]; glycosidases, *e.g.* neuraminidase on the surface of influenza virus[123]; proteases, *e.g.* metalloproteases MMP9 and MMP14[124] and integrins, dendritic cell surface bound αvβ8 and epithelial cell surface bound αvβ6 for activating TGF-β for T cells and macrophages respectively[107,125,126].

Despite these complex interactions, the basic principle of TGF-β maturation is simple: an enzymatic digestion- or interaction-based conformational change that switches the latent complex into an open form to free the TGF-β dimer. Such a process can be highly content specific, owing to the different, overlapping or even contradictory regulators that exist in the whole system. For example Fibrillin 1 anchors LTBPs to fibers in the extracellular matrix and inhibits TGF-β activation in certain tissues. Mutations found in LTBPs suppress TGF-β ligand activation in lung and bone development. On the contrary, mutations in human Fibrillin 1 lead to excessive TGF-β activation in lung and aortic aneurysms[119]. As another example, glycoprotein A

repetitions predominant protein (GARP), is found to be restricted to Foxp3^{+} Tregs and counteracts the binding of LTBP to TGF-β[127].

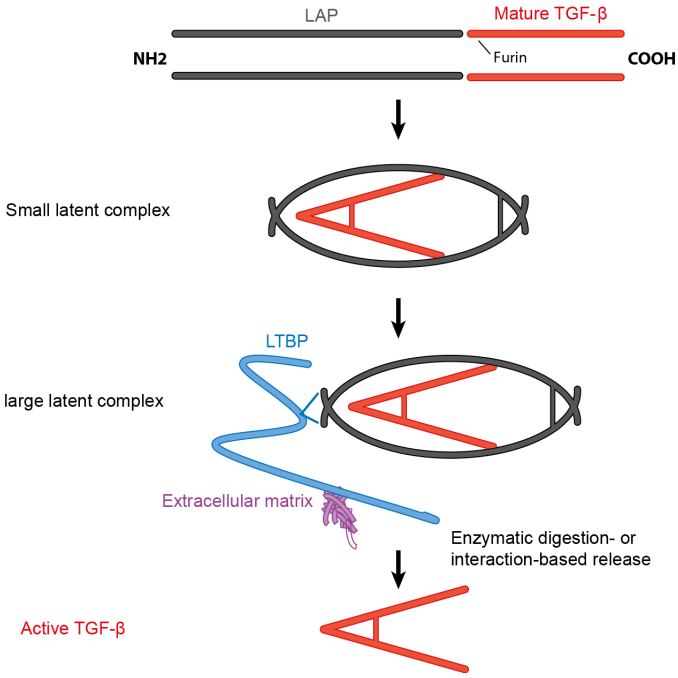

Figure 5. Processing and activation of TGF-β. *Tgfb* genes encode an N-terminal latency-associated peptide (LAP) and a C-terminal mature cytokine that form dimers after being synthesized. The LAP and the mature TGF-β cytokine are cleaved from each other by the enzyme furin but remain non-covalently associated after secretion. The LAP region folds around the mature cytokine, blocking its access to the receptors, and this is termed the small latent complex. In some cells, the small latent complex can associate with latent TGF-β binding protein (LTBP) via interactions with LAP, forming the large latent complex which facilitates the interaction with extracellular matrix. Once the LAP has been digested enzymatically or removed via interaction-based conformational change, the mature TGF-β is released and becomes active for receptor binding. Adapted from reference[119].

4.4.1.2 The divergence in TGF-βR signaling transduction

After being released from the latent complex, TGF-β ligand binds exclusively to TGF-βR2 and vice versa. Evidence exists that instead of TGF-βR1, in certain cell types or with high TGF-β concentration, TGF-βR2 could bind and activate ALK1 or ALK2, both BMP family receptors, which lead to the phosphorylation and activation of Smad1/5 and subsequently the bifurcation into the BMP signaling pathway[118].

TGF-β can signal without Smad-mediated transcriptional regulation, that is, in a non-canonical fashion. For example, in non-canonical TGF-βR signaling pathway, phosphorylated TGF-βR directly regulates signaling events in MAPK/ERK pathways and PI3K/AKT pathways in a Smad-independent manner, as one of the key mediators

in cell growth, proliferation and survival[118]. Also, TGF-β could directly signal through TGF-βR2 via phosphorylation of the cell polarity regulator, partitioning defective 6 (PAR6), in epithelial to mesenchymal transition for RhoA GTPase degredation or in axon formation in neurons[128,129]. Further, pSmad3 could serve as a Drosha component in promoting the biogenesis of microRNAs that harbor Smad binding sites[130,131]. (Figure 6.)

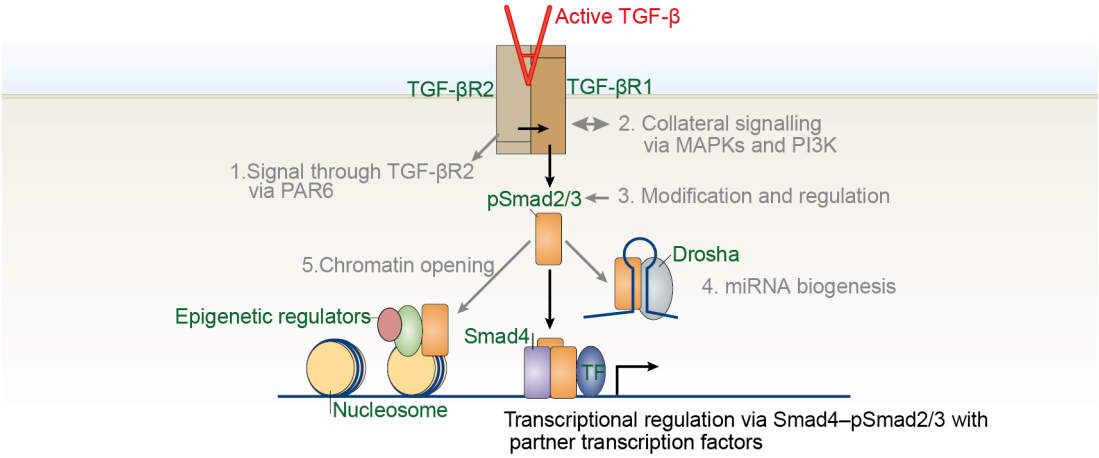

Figure 6. The canonical TGF-βR signaling and its divergence and regulation. In canonical TGF-βR signaling (indicated by black arrows), the binding of active TGF-β to TGF-βR2 dimers induces the phosphorylation and activation of TGF-βR1 dimers, which bind to and phosphorylate Smad2 or Smad3. Phosphorylated Smad2/3 (pSmad2/3) bind to Smad4 and, as a complex, they translocate to the nucleus and initiate transcription regulation of target genes together with other transcription factors. Dependent on the status of the cell, many other signaling events can happen (indicated by grey arrows): 1. signaling directly through TGF-βR2 via PAR6; 2. signaling via MAPKs and PI3K pathways independent of Smads proteins; 3. variations of the signaling activity due to regulations and modifications on Smad proteins; 4. controlling miRNA biogenesis via Smad protein directed Drosha complex assembly; 5. Smad protein associated epigenetic regulators recruitment. Adapted from reference[118].

4.4.1.3 Regulators and transcription initiation

The activity of pSmad itself is highly modulated by ubiquitination-mediated degradation, kinase activity, and other modifications, and serves as a key target in regulating TGF-βR signaling. For example, Smads can be ubiquitinated and degraded via Smad7-mediated Smurf recruitment, with competition from deubiquitases like USP15[132,133]. In response to other growth factors, extracellular stress and cell intrinsic status, components from ERK/MAPK signaling and the cell cycle are integrated into TGF-βR signaling through modification of pSmad[118].

In the canonical pathway, Smad proteins not only influence the transcription of active genes via cooperations with diverse sets of transcription factors and cofactor, but they also shape the epigenetic state of target genes. Smad proteins recruit histone acetyl transferases for general transcription activation. Through binding the partners TGIF1/2 mediated C-terminal-binding protein and histone deacetylases recruitment, Smad proteins suppress gene accessibility via histo-deacetylation. With TGF-β stimulation, locus of gene *CDNK2B* is de-methylated at its promoter region and becomes active after SMAD4-SMAD2/3-FOXO-mediated DNA excision repair complex recruitment[118,134,135].

Overall, the response of a given cell type to TGF-βR signaling is determined by defined input signals and the compositions of signaling network machinery in that cell, combinations of transcription factors, cofactors and epigenetic regulators that are actually expressed in this particular cell type and their interaction with the Smad complex, and further the established general epigenetic status of the cell and the accessibility of a given locus, that is, whether the chromatin is open for transcriptional regulation initiated by TGF-βR signaling.

4.4.2 The Role of TGF-βR Signaling in the Immune System

TGF-β ligands are widely and highly expressed during embryogenesis in a cell-specific and time-specific manner and they are also detectable in adult in the steady state. Of the three highly homologous TGF-β isoforms (TGF-β1, TGF-β2, TGF-β3), TGF-β1 is the predominant isoform within the immune system[118,136,137].

The importance of TGF-β signaling in embryogenesis and its regulatory role in the immune system has been revealed from early studies on *Tgfbr2*$^{-/-}$ mice and *Tgfb1*$^{-/-}$ mice. *Tgfbr2*$^{-/-}$ embryos and one half of the *Tgfb1*$^{-/-}$ embryos die at E10.5 due to hematopoietic and vasculo-genetic defects in the yolk sac[138,139]. The other half of *Tgfb1*$^{-/-}$ embryos survive until a few weeks after birth and then die because of a wasting syndrome and multifocal inflammatory disease[138,140,141]. It was shown that TGF-β is responsible for NK cell immaturity during their ontogeny[142]. Studies on T cells suggest that TGF-β signaling affects many aspects of T cells, for example on CD4+ and CD8+ T cell survival, CD8+ T cell, NKT cell and nTreg cell development, and Th1, Th2, Th17 and iTreg differentiation[119,143].

TGF-βR signaling is widely involved in stem cell function and differentiation. Within the hematopoietic system, TGF-βR signaling maintains HSCs in quiescence and hence prevents their exhaustion. During the early differentiation of HSCs, TGF-βR signaling is a stimulant for myeloid-biased HSC proliferation as well as an inhibitor of lymphoid-biased HSC[144]. For the genesis of yolk sac derived precursors, the role of TGF-βR signaling remains largely unknown. Due to lethality in TGF-βR signaling deficient mice, the exact function of TGF-βR signaling cannot be identified from the large influence from its impact on organogenesis.

Regarding the myeloid system, TGF-βRs are broadly expressed by DCs and macrophages. According to the ImmGen data[53], *Tgfbr1* and *Tgfbr2* are expressed at diverse levels on different DC subsets and tissue-resident macrophage populations. For example LCs, microglia, AMs, small intestine lamina propria and serosal macrophages and kidney $CD11b^+$ DCs exhibit high *Tgfbr2* expression. Mice in which *Tgfbr2* was specifically deleted from DCs ($CD11c^{Cre}Tgfbr2^{fl/fl}$) showed an altered Treg phenotype and multi-organ autoimmune disease caused by 'proinflammatory' DCs. This reveals a direct effect of TGF-β signaling on DCs to control autoimmunity[145]. Recently, studies have shown that TGF-βR signaling regulates $CD103^+CD11b^+$ DC development in the intestine, indicating a role of TGF-β in DCs under the steady state condition. The development and maintenance of LCs, the resident embryonically-derived myeloid cells of the skin, is dependent on TGF-βR signaling[92,93].TGF-β stimulation allows PU.1 to directly induce Runx3 and Id2, and the expression of the latter prevents B cell-related gene expression in a progenitor cell.[144,146]. TGF-β has also been shown to be critical for microglial development and influences microglia gene signature expression and activation *in vitro* and *in vivo*[147,148].

5 Aims

In the past few years, great effort has been made to uncover signature genes, unique transcriptional factors and enhancer landscapes of different tissue-resident macrophages. However, extracellular signaling mediators that govern the differential gene expressions during macrophage development are largely unknown. TGF-β is a cytokine with versatile effects on cell fate determination. Therefore, we aimed to investigate the role of TGF-β across different tissue-resident macrophage populations during their development, maturation and homeostasis.

Specifically, we focused on unraveling the role of TGF-βR signaling in alveolar macrophages (AMs), as they highly express TGF-βRs in the steady state. Using mouse models with different Cre recombinase-dependent *Tgfbr2* locus deletion, we determined the role of TGF-βR signaling on AMs in their differentiation, maturation, self-maintenance and function in the steady state. In addition, we aimed to identify the source of TGF-β that is required by AMs in these processes.

Furthermore, we investigated the downstream effects of TGF-βR signaling in AMs. By comparing gene expression levels in TGF-βR deficient AMs versus intact AMs, we aimed to gain insight into the relationship among AM-specific genes, the 'AM cytokine' GM-CSF and TGF-βR signaling.

6 Results

6.1 AMs are Dependent on TGF-βR Signaling

To investigate the role of TGF-βR signaling in the development of tissue macrophages, we analyzed *Itgax*^{Cre}*Tgfbr2*$^{fl/fl}$ mice, in which *Tgfbr2* is deleted in CD11c^{+} cells including DCs and macrophages in the lung, the small intestinal lamina propria and the kidney. We found that at 4 weeks of age, AMs were completely absent in *Itgax*^{Cre}*Tgfbr2*$^{fl/fl}$ mice (Figure 7A-B). Conversely, lung interstitial macrophages (CD11c^{+}MHCII^{+}CD11b^{+}CD24^{-}CD64^{+})[149] and DCs were not affected (Figure 7B-C). Other CD11c^{+} tissue macrophages including kidney and small intestinal lamina propria macrophages, were also present at normal frequencies in *Itgax*^{Cre}*Tgfbr2*$^{fl/fl}$ mice, while LCs were largely absent, consistent with their known dependence on TGF-βR signaling (Figure 8A). *Itgax*^{Cre}*Tgfbr2*$^{fl/fl}$ mice develop multi-organ autoimmune disease due to deletion of *Tgfbr2* on DCs starting at the age of 4-5 weeks[145]. Indeed, at 4 weeks of age we already observed increased invasion of monocytes and neutrophils into most tissues in *Itgax*^{Cre}*Tgfbr2*$^{fl/fl}$ mice (data not shown). Thus, to rule out that the observed AM phenotype is a result of local inflammation, analyses were performed at earlier time points at which the *Itgax*^{Cre}*Tgfbr2*$^{fl/fl}$ mice appear clinically and phenotypically healthy. We found that at two weeks of age, AMs were already absent (Figure 7A).

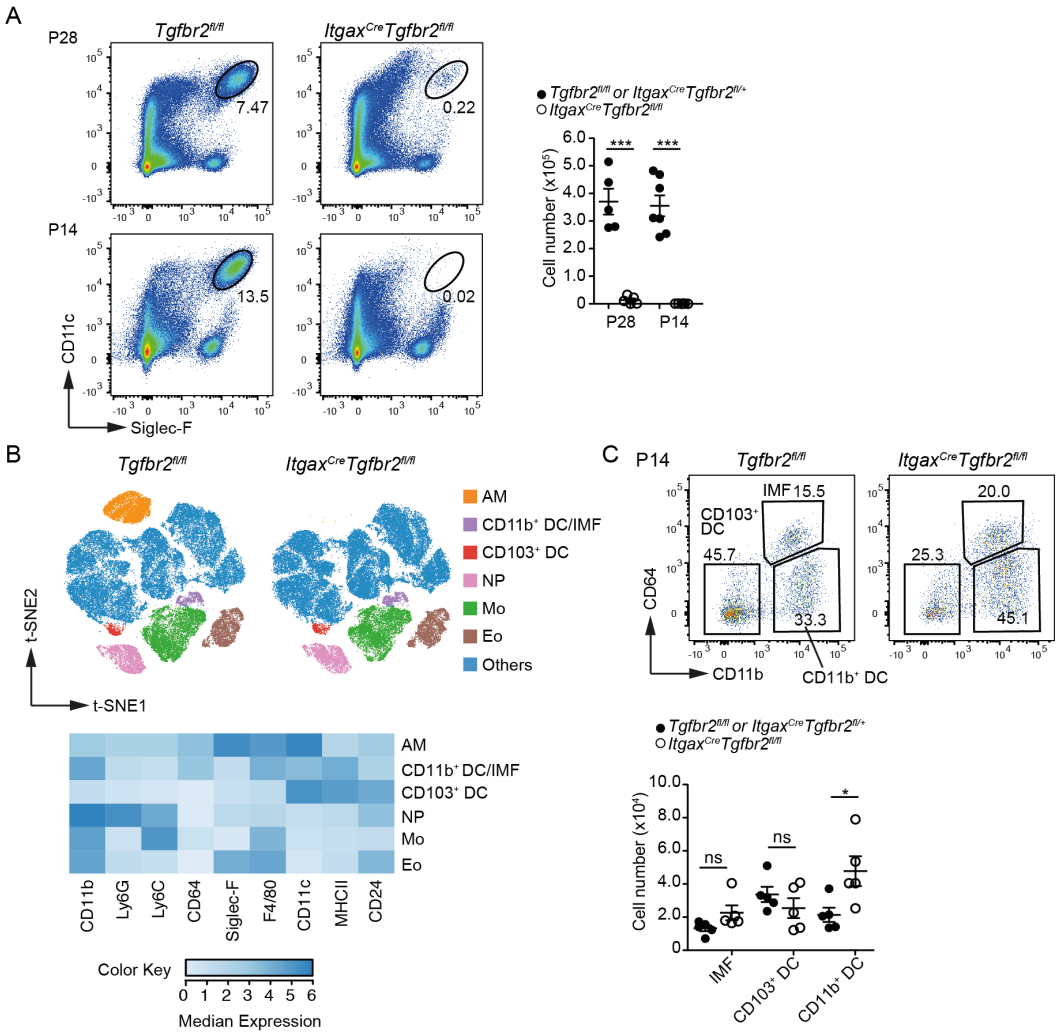

Figure 7. AMs are dependent on TGF-βR signaling. (A-C) Flow cytometry of lung cells derived from *Itgax*^{Cre}*Tgfbr2*$^{fl/fl}$ mice and control littermates (*Tgfbr2*$^{fl/fl}$ or *Itgax*^{Cre}*Tgfbr2*$^{fl/+}$) at postnatal day (P)28 and P14. Representative flow cytomety plots and graphs (±SEM) show the frequency and total cell numbers of AMs (Siglec-F$^+$CD11c$^+$, pre-gated on CD45$^+$ cells) (A) at P14 and P28 and CD103$^+$ DCs, CD11b$^+$ DCs and interstitial MFs (IMF) (pre-gated on CD45$^+$Ly6G$^-$Siglec-F$^-$Ly6C$^-$ MHCII$^+$CD11c$^+$) at P14 (C). N = 4-11 from 2-3 independent experiments (A) and N = 5 from 2 independent experiments (C). *p < 0.05, **p < 0.01, ***p < 0.001 (Student's t test, unpaired). **(B)** Annotated t-SNE plots and expression of markers in the identified populations among CD45$^+$ lung cells from *Tgfbr2*$^{fl/fl}$ or *Itgax*^{Cre}*Tgfbr2*$^{fl/fl}$ mice (P14) as in (A). Archsinh transformed medians are shown.

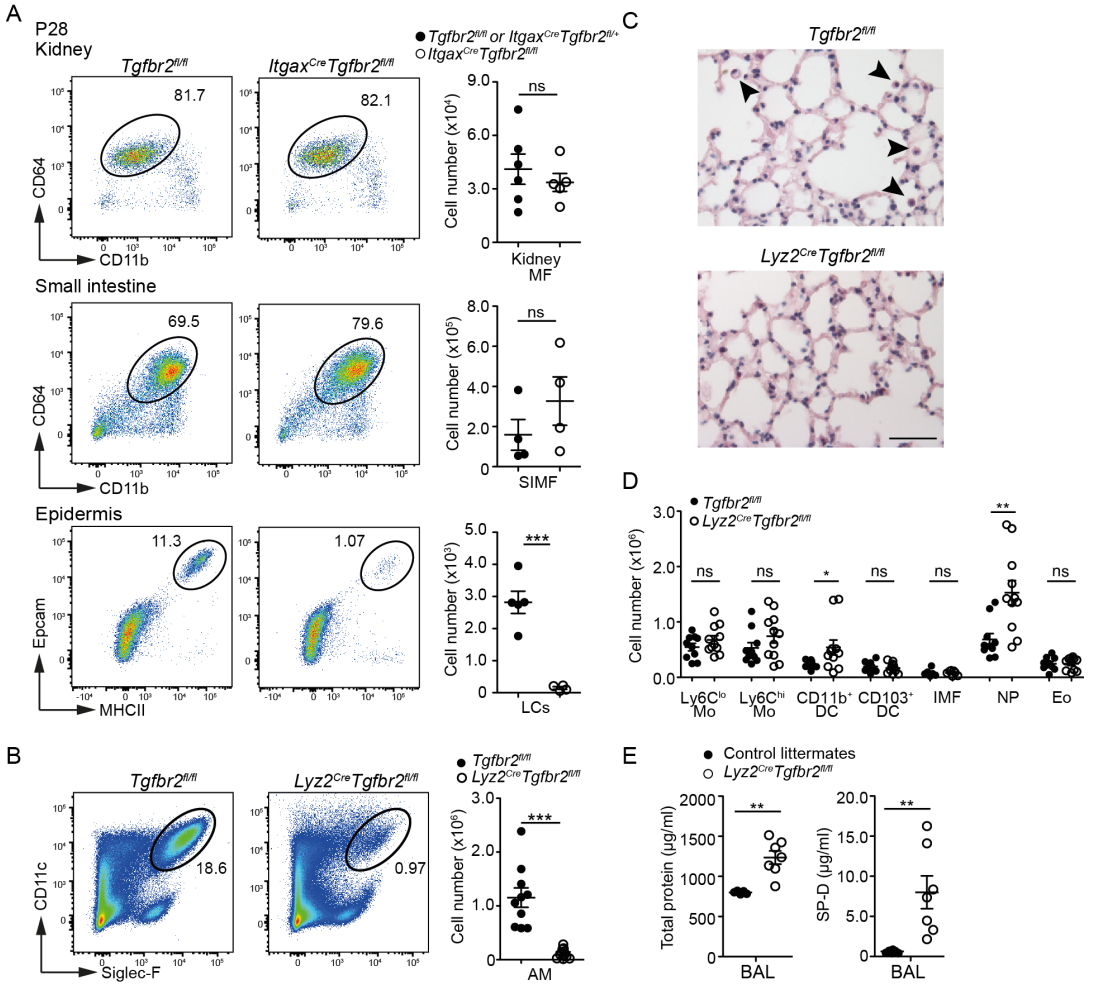

Figure 8. TGF-βR signaling is not crucial for other tissue macrophages in *Itgax^{Cre}Tgfbr2$^{fl/fl}$* and *Lyz2^{Cre}Tgfbr2$^{fl/fl}$* mice. (A) Flow cytometry (left) and quantification (right) (±SEM) of kidney macrophages (Kidney MF), small intestinal lamina propria macrophages (SIMFs) and LCs of *Itgax^{Cre}Tgfbr2$^{fl/fl}$* mice and control littermates (*Tgfbr2$^{fl/fl}$* or *Itgax^{Cre}Tgfbr2$^{fl/+}$*) at P28. Kidney MFs and SIMFs were pre-gated on CD45$^+$Ly6G$^-$Siglec-F$^-$Ly6C$^-$MHCII$^+$CD11c$^+$ cells. LCs were pre-gated on CD45$^+$Ly6G$^-$ cells. N = 4-6, from 2-3 independent experiments. **(B)** Flow cytometry of lungs of adult *Lyz2^{Cre}Tgfbr2$^{fl/fl}$* and *Tgfbr2$^{fl/fl}$* mice. Flow cytometry plots show the frequency of AMs (Siglec-F$^+$CD11c$^+$, pre-gated on CD45$^+$) (left) and quantification of total cell numbers (±SEM) of AMs (right). N = 9-11, from 3 independent experiments. **(C)** H&E staining of lungs from *Lyz2^{Cre}Tgfbr2$^{fl/fl}$* and *Tgfbr2$^{fl/fl}$* mice. Scale bar = 40 µm. **(D)** Quantification of total cell numbers (±SEM) of Ly6C^{hi} Mo, Ly6C^{lo} Mo, CD11b$^+$ DCs, CD103$^+$ DCs, IMFs, NPs and Eos of lungs from *Lyz2^{Cre}Tgfbr2$^{fl/fl}$* mice and from control littermates by flow cytometry. (Gating strategy shown in Figure S1A). N ≥ 6, from 2-3 independent experiments. **(E)** Total protein concentration (left) and SP-D protein concentration (right) (±SEM) in the BAL from *Lyz2^{Cre}Tgfbr2$^{fl/fl}$* (either *Lyz2$^{Cre/Cre}$Tgfbr2$^{fl/fl}$* or *Lyz2$^{Cre/+}$Tgfbr2$^{fl/fl}$*, 6-8 weeks old) and from control littermates (*Lyz2$^{Cre/Cre}$Tgfbr2$^{fl/+}$*, *Lyz2$^{Cre/+}$Tgfbr2$^{fl/+}$* or *Tgfbr2$^{fl/fl}$*), N ≥ 6. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant (unpaired Student's t test).

To determine the differential impact of TGF-βR signaling across the MPS, we next generated *Lyz2^{Cre}Tgfbr2$^{fl/fl}$* mice, in which *Tgfbr2* is deleted in lysozyme-expressing myeloid cells including macrophages, neutrophils and monocytes. Analysis of adult *Lyz2^{Cre}Tgfbr2$^{fl/fl}$* mice revealed that in the lung, AMs were essentially absent, whereas the numbers of monocytes, DCs and interstitial macrophages were again unaltered

(Figure 8B-D). Also, in other non-lymphoid and lymphoid tissues, macrophages were present at normal frequencies in *Lyz2*^{Cre}*Tgfbr2*$^{fl/fl}$ mice (data not shown). Concomitant with the absence of AMs, we found that the total protein conentration and surfactant protein D (SP-D) concentration increased in the bronchoalveolar lavage (BAL) of *Lyz2*^{Cre}*Tgfbr2*$^{fl/fl}$ mice (Figure 8E). This is indicative of the development of PAP as previously shown in mice lacking AMs[60,94]. However, in *Lyz2*^{Cre}*Tgfbr2*$^{fl/fl}$ mice (heterozygous for Cre), the numbers of AMs often recovered with age (data not shown). In these AMs, *Tgfbr2* expression was comparable to WT levels suggesting a repopulation by TGF-βR-sufficient precursors and/or AMs over time. Conversely, by increasing the recombination frequency in *Lyz2*$^{Cre/Cre}$*Tgfbr2*$^{fl/fl}$ mice (homozygous for Cre), AMs remained absent (data not shown) but these mice died at variable ages most likely due to multi-organ inflammation[145,150].

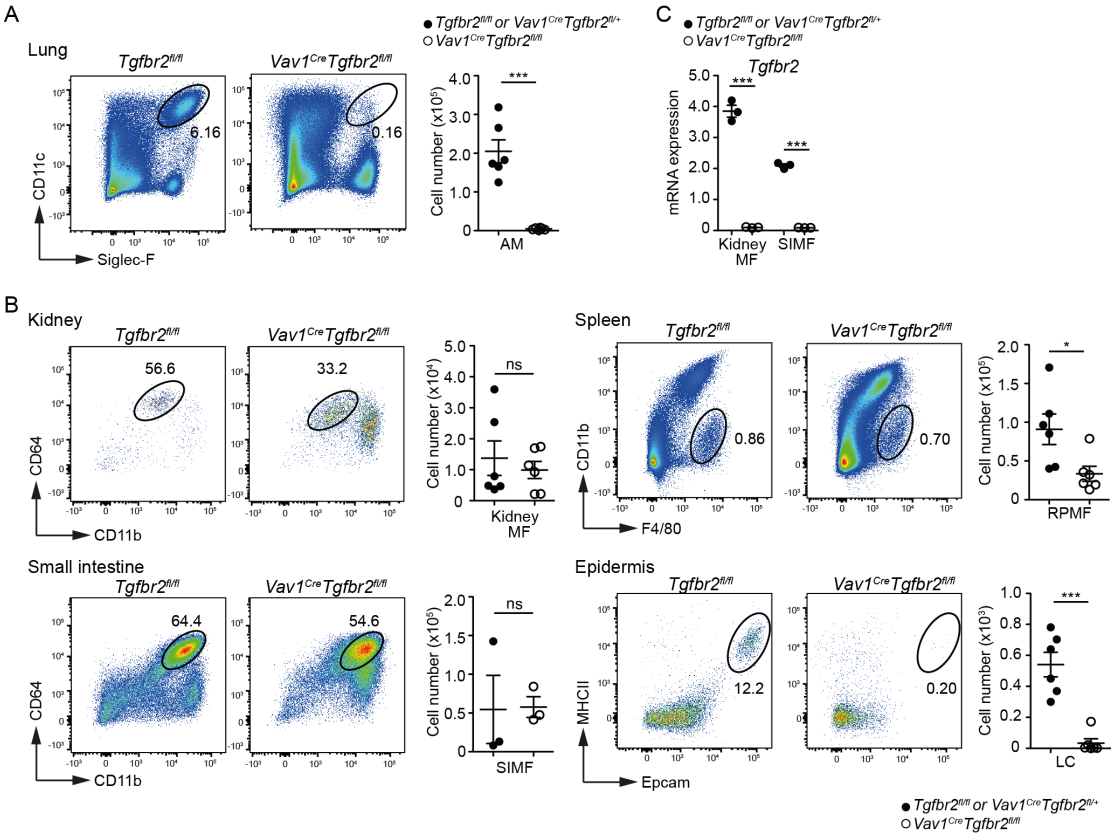

Figure 9. TGF-βR signaling is not crucial for other tissue macrophages in *Vav1*^{Cre}*Tgfbr2*$^{fl/fl}$ mice. **(A-B)** Flow cytometry (left) and quantification (right) (±SEM) of AMs (A), kidney MFs, SIMFs, RPMFs and LCs (B) of *Vav1*^{Cre}*Tgfbr2*$^{fl/fl}$ mice and control littermates (*Tgfbr2*$^{fl/fl}$ or *Vav1*^{Cre}*Tgfbr2*$^{fl/+}$) at P14. N = 3, from 2 independent experiments. **(C)** Relative mRNA expression levels (±SEM) of *Tgfbr2* from sorted kidney MFs and SIMFs as shown in (B), normalized to *Pol2*. N = 3. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant (unpaired Student's t test).

Lyz2^{Cre} mice display varying degrees of recombination efficiency across the MPS[112,151]. To definitively target all members of the MPS, we analyzed 2-week-old *Vav1*^{Cre}*Tgfbr2*$^{fl/fl}$ mice, in which *Tgfbr2* is deleted in all hematopoietic cells[152]. Also here, while AMs and LCs were absent, other macrophage populations analyzed were unaffected with the exception of red pulp macrophages, which were 50% reduced (Figure 9A-B). Successful deletion of *Tgfbr2* was verified by RT-PCR (Figure 9C). Taken together, TGF-βR signaling is essential for the development and/or maintenance of AMs but is not required for the genesis of other tissue macrophages.

6.2 Autocrine TGF-β is Required for AMs

We found TGF-β to be highly expressed in the adult lung as previously shown[153,154] (Figure 10A). Within the lung, we detected its expression in epithelial cells, endothelial cells and AMs, with the highest expression in AMs (Figure 10B). TGF-β can be activated from its latent form in a cell-cell contact-dependent manner through the integrin avβ6 expressed by the lung epithelium[155]. On the other hand, it was shown *in vitro* that TGF-β can be activated by AMs in an autocrine manner by the interaction with thrombospondin 1, CD36 and the protease plasmin[156]. To evaluate whether TGF-β regulates AMs in an autocrine manner *in vivo,* we analyzed *Itgax*^{Cre}*Tgfb1*$^{fl/fl}$ mice in which *Tgfb1* is deleted in CD11c^{+} cells. At 4 weeks of age, the number and frequency of AMs derived from whole lung tissue or from the BAL were significantly reduced in *Itgax*^{Cre}*Tgfb1*$^{fl/fl}$ mice (Figure 10C-D). The remaining cells from the BAL exhibited a foam-cell-like phenotype, characterized by enlarged cytoplasm and lipid accumulation as assessed with Oil Red O staining (Figure 10E). Collectively, these data suggest that AMs require TGF-β for their development and/or maintenance in an autocrine manner.

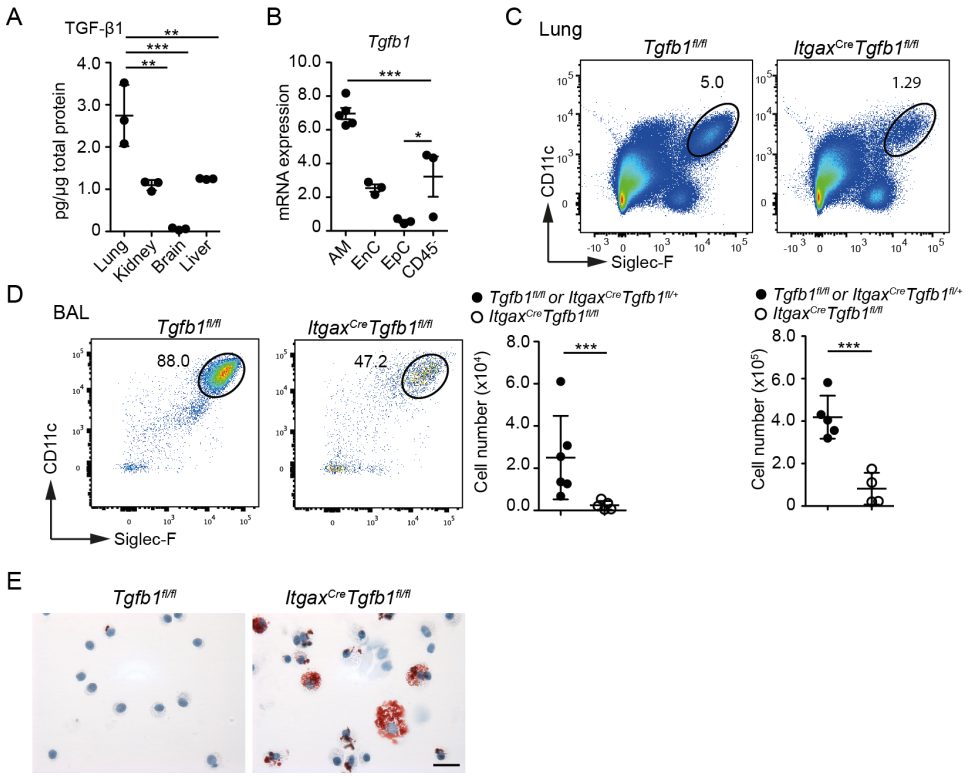

Figure 10. TGF-β is required for AMs in an autocrine manner. (A) ELISA for TGF-β1 of the lung, liver, kidney and brain from WT mice, normalized to total protein amount. N = 3. **(B)** Relative mRNA

expression of *Tgfb1* from sorted AMs, epithelial cells (EpC, pre-gated on $CD45^-Epcam^+CD31^-$), endothelial cells (EnC, pre-gated on $CD45^-Epcam^-CD31^+$) and other $CD45^-$ cells from WT lungs, normalized to *Pol2*. N ≥ 3. **(C-D)** Representative flow cytometry plots of AMs ($Siglec\text{-}F^+CD11c^+$) among $CD45^+$ cells from lung tissue (C) or from bronchoalvelar lavage (BAL) (D) from $Itgax^{Cre}Tgfb1^{fl/fl}$ mice and control littermates ($Tgfb1^{fl/fl}$, $Tgfb1^{fl/+}$ or $Itgax^{Cre}Tgfb1^{fl/+}$) and quantification of total cell numbers (±SEM) on the right. N ≥ 4 from 2 independent experiments. **(E)** Oil Red O staining of AMs derived from the BAL of $Itgax^{Cre}Tgfb1^{fl/fl}$ mice and from control littermates. Scale bar = 20 μm. Representative images are shown. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant (unpaired Student's t test for (C-D) and one-way ANOVA for (A-B)).

6.3 TGF-βR Signaling is Essential for the Maintenance of Mature AMs

To investigate the impact of TGF-βR signaling on adult AM homeostasis, we crossed the *Tgfbr2*$^{fl/fl}$ mice to the tamoxifen-inducible *Rosa26*^{CreER} (*R26*^{CreER}) mice, in which tamoxifen administration leads to deletion of *Tgfbr2* in all cells and tissues. 6-8 week old *R26*^{CreER}*Tgfbr2*$^{fl/fl}$ mice were treated with tamoxifen every other day for 5 times and AMs were analyzed 7 days after the last treatment. Deletion of TGF-βR in mature AMs led to a significant reduction of their numbers (Figure 11A). The remaining AMs were found to be untargeted (Figure 11B), demonstrating that TGF-βR signaling on AMs is important for their maintenance.

Again, to address whether, in addition to AMs, other tissue macrophages also depend on TGF-βR signaling for their homeostatic maintenance, we used *Cx3cr1*^{CreER}*Tgfbr2*$^{fl/fl}$, in which tamoxifen administration leads to the specific deletion of TGF-βR in cells expressing the chemokine receptor CX_3CR1^+, which includes microglia, kidney and gut macrophages, but not AMs[40]. As recently demonstrated[157], tamoxifen-inducible deletion of *Tgfbr2* in adult microglia led to rapid microglia activation but not to their depletion. TGF-βR ablation did not alter the frequency of gut and kidney macrophages in *Cx3cr1*^{CreER}*Tgfbr2*$^{fl/f}$ mice compared to control littermate controls (Figure 11C-D). Successful *Tgfbr2* deletion in these populations was confirmed by RT-PCR (Figure 11E). In contrast to microglia[157], deletion of TGF-βR in gut and kidney macrophages did not trigger their transformation into inflammatory macrophages in the time frame analyzed as assessed by *Il1b* and *Tnf* cytokine expression (Figure 11E). Together, these data indicated that the homeostasis of AMs required tonic TGF-βR engagement, whereas the survival of microglia, gut and kidney macrophages was not dependent on TGF-βR signaling.

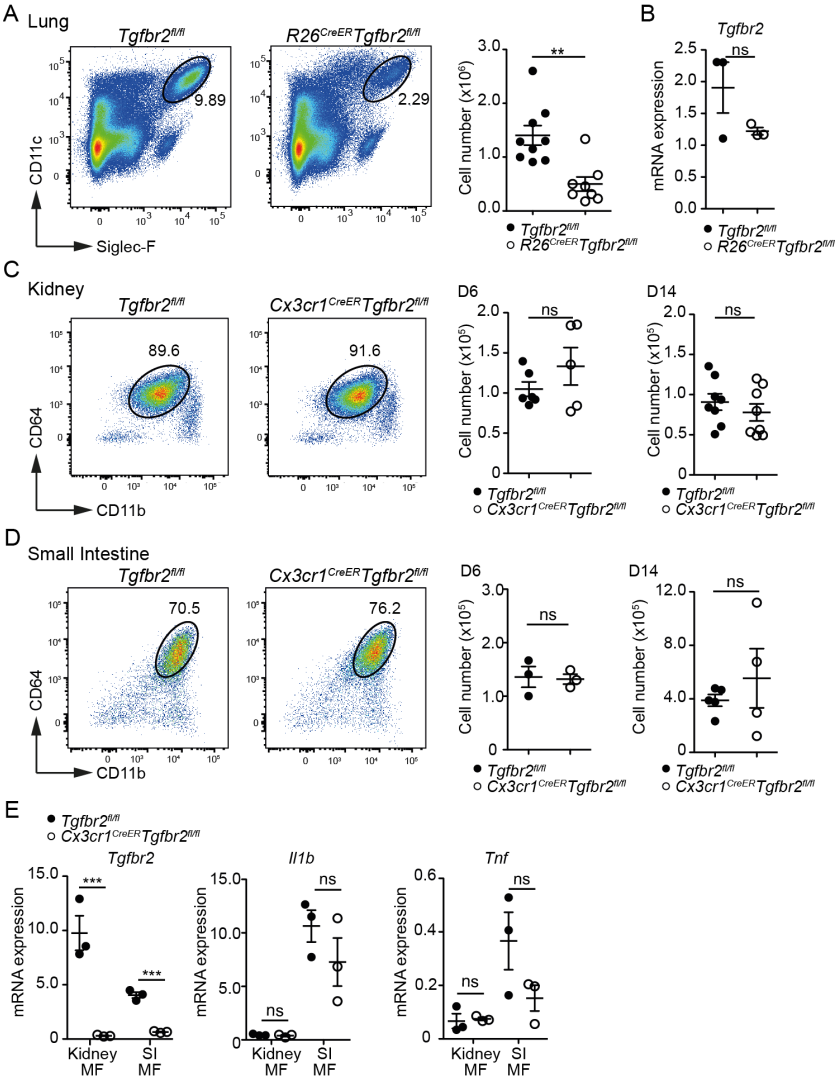

Figure 11. TGF-βR signaling is required for the homeostasis of AMs. **(A-B)** *R26*^{CreER}*Tgfbr2*$^{fl/fl}$ mice and *Tgfbr2*$^{fl/fl}$ littermates were treated with tamoxifen (5 mg) 5 times every other day via oral gavage (o.g.) and analyzed 7 days after the last treatment. **(A)** Flow cytometry (left) of AMs (Siglec-F$^+$CD11c$^+$) (pre-gated on CD45$^+$ cells) and quantification (right) of total cell numbers (±SEM). N ≥ 9 from 4 independent experiments. **(B)** qRT-PCR of *Tgfbr2* mRNA from AMs (Siglec-F$^+$CD11c$^+$) as shown in (A), normalized to *Pol2* expression. N = 3. **(C-E)** *Cx3cr1*^{CreER}*Tgfbr2*$^{fl/fl}$ mice and *Tgfbr2*$^{fl/fl}$ littermates were treated with tamoxifen every other day (max. 5 times) via o.g. and analyzed 6 and 14 days after treatment start. Representative flow cytometry plots (left) on D6 and quantification of total cell numbers (right) on D6 and D14 of kidney macrophages (MF) (C) and small intestinal lamina propria macrophages (SI MF) (D) (pre-gated on CD45$^+$Ly6G$^-$Siglec-F$^-$Ly6C$^-$MHCII$^+$CD11c$^+$ cells). N ≥ 3 from 2-8 independent experiments. **(E)** qRT-PCR of *Tgfbr2, Il1b and Tnf* mRNA from kidney MFs and SIMFs at D6, normalized to *Pol2* expression. N = 3. *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant (unpaired Student's t test).

It has been shown that activation of TGF-βR signalling in AMs might be associated with the adherence of these cells to lung epithelial cells[158]. To address whether it is the mechanism by which AMs require TGF-βR signalling for their maintenance, we have treated adult *R26*^{CreER}*Tgfbr2*$^{fl/fl}$ and *Tgfbr2*$^{fl/fl}$ mice with tamoxifen every other

day and analysed the BAL and lung tissue 6 days after the first treatment. Even though *Tgfbr2* deletion was already evident at D6 post tamoxifen treatment, this did not significantly alter the number of AMs in the BAL (Figure 12A-C). Likewise, using immunohistochemistry, no significant difference in adherent vs. non-adherent AMs was detected (Figure 12D). This is indicative that the loss of AMs upon removing tonic TGF-βR signaling leads to cell-intrinsic AM loss. As to whether the dead AMs are then absorbed *in situ* or accumulate in the BAL cannot be formally demonstrated or excluded. Therefore, the data we accumulated thus far do not support the notion that the loss of AMs upon disruption of TGF-βR signaling is caused by the detachment from epithelial cells.

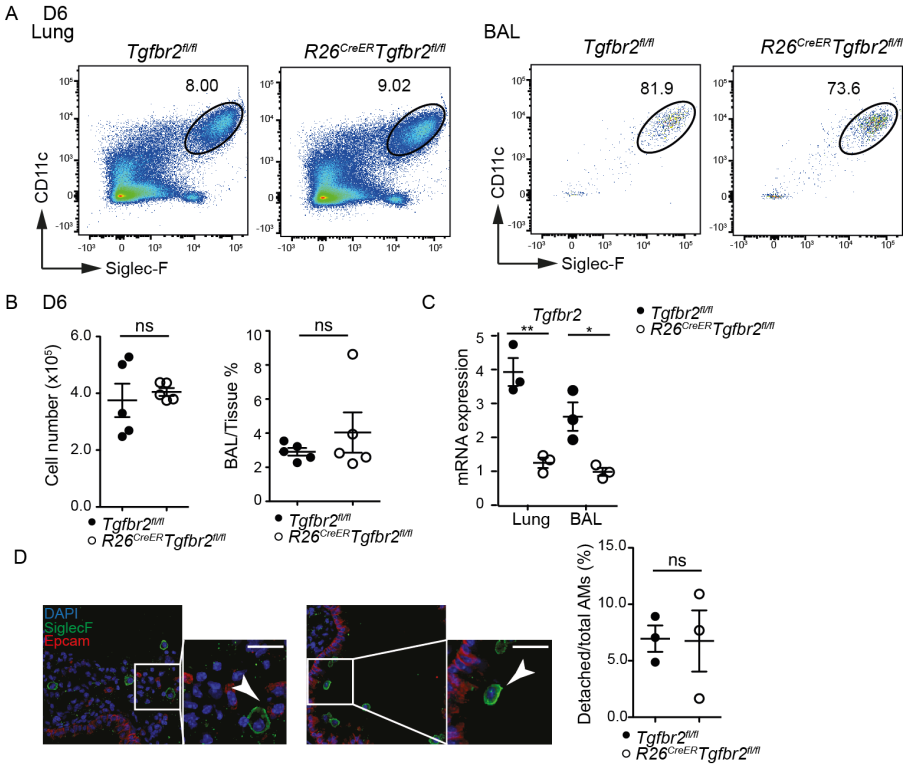

Figure 12. Disruption of TGF-βR signaling is not associated with AM detachment from epithelial cells. (A-D) *R26*^{CreER}*Tgfbr2*$^{fl/fl}$ and *Tgfbr2*$^{fl/fl}$ mice were treated with tamoxifen (5 mg) 3 times every other day via oral gavage and analyzed 2 days after the last treatment (D6 after treatment start). **(A)** Flow cytometry of AMs (Siglec-F^{+}CD11c^{+}, pre-gated on CD45^{+} cells) from lung tissue and BAL. Representative images. **(B)** Quantification of total cell numbers of AMs in the lung tissue (left) and the ratio of AMs in the BAL vs. lung tissue (right). **(C)** qRT-PCR of *Tgfbr2* mRNA from AMs as shown in (A), normalized to *Pol2* expression. **(D)** Representative image of an AM attached to lung epithelium (top images) and a 'detached' AM (bottom images) by IHC of the lung, stained for Siglec-F (green), DAPI (blue) and Epcam (red). Arrowheads indicate AMs. Scale bars, 20 µm. Dot plots shows the ratio of detached vs. total AMs. Each dot represents at least 50 AMs of ≥ 2 mice. (A-B), N = 5. ±SEM. ns, not significant (unpaired Student's t test). (C) N = 3. ±SEM. *p < 0.05, **p < 0.01(unpaired Student's t test).

6.4 The Development of Monocyte-Derived 'AMs' after Irradiation Requires TGF-βR Signaling

AMs self-maintain in the steady state independently of circulating precursors (Hashimoto et al., 2013). However, in inflammation or after lethal whole body irradiation and subsequent BM transplantation, BM-derived monocytes repopulate the AM niche[58]. To investigate whether in BM transplantation, those BM-derived 'AMs' are also dependent on TGF-βR signaling, BM chimeric mice were generated with a 1:1 mix of WT (CD45.1) and *Lyz2*^{Cre}*Tgfbr2*$^{fl/fl}$ (CD45.2) BM. *Csf2rb*$^{-/-}$ (CD45.2) or WT (CD45.2) BM served as controls. This setup allowed for the investigation of the intrinsic requirements for TGF-βR signaling for the development of BM-derived 'AMs' with a competing internal WT control population. We found all AMs to be of WT origin whereas blood monocytes were reconstituted at a 1:1 ratio in *Lyz2*^{Cre}*Tgfbr2*$^{fl/fl}$: WT chimeras (Figure 13). This finding is reminiscent of what is observed when GM-CSFR signaling is disturbed[58,90,94] (Figure 13) These results demonstrate that TGF-βR is not only important for embryonically-derived AMs but also for the repopulation of AMs from adult BM after total body irradiation.

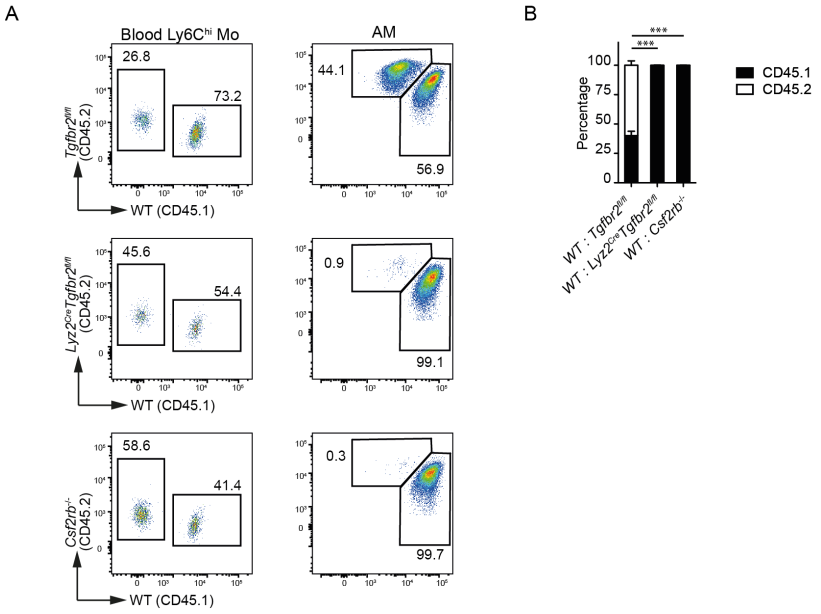

Figure 13. BM-derived 'AMs' are dependent on TGF-βR signaling. (A) Representative flow cytometry plots depict the frequency of CD45.1$^+$ and CD45.2$^+$ blood Ly6C^{hi} monocytes (pre-gated on F4/80$^+$CD115$^+$CD11b$^+$Ly6C^{hi}Ly6G$^-$ cells) and AMs (pre-gated on Siglec-F$^+$CD11c$^+$ cells) in mixed WT(CD45.1$^+$) : *Lyz2*^{Cre}*Tgfbr2*$^{fl/fl}$ (CD45.2$^+$), WT(CD45.1$^+$) : *Tgfbr2*$^{fl/fl}$(CD45.2$^+$) and WT(CD45.1$^+$) : *Csf2rb*$^{-/-}$(CD45.2$^+$) BM chimeras. Remaining host cells (CD45.1$^+$CD45.2$^+$) were gated out. **(B)** Quantification (±SEM) of (A) normalized to the ratio of CD45.1$^+$ and CD45.2$^+$ blood Ly6C^{hi} Mo. N = 3-6, from 2-3 independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant (unpaired Student's t test).

6.5 Embryonic Development of AMs Depends on TGF-β

In order to define whether TGF-β signaling is a prerequisite for the embryonic development of AMs, we first analyzed the expression of *Tgfb1* and its receptor in preAMs and fetal monocytes. We found the expression of *Tgfb1* in fetal monocytes and preAMs was comparable to mature AMs, whereas *Tgfbr2* and *Tgfbr1* were more highly expressed in adult AMs compared to preAMs and fetal lung monocytes (Figure 14).

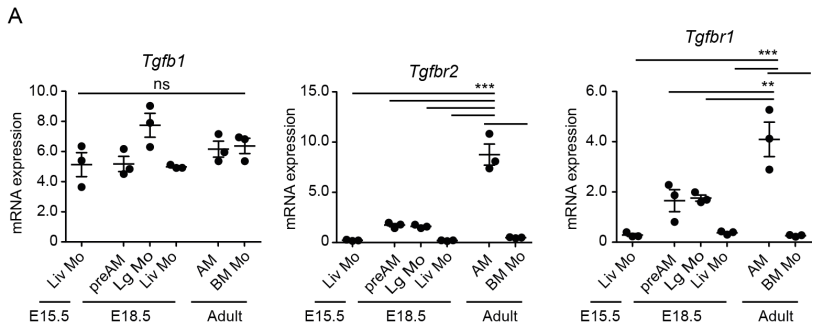

Figure 14. TGF-βRs and ligand expression in AMs and precursors (A) Relative mRNA expression levels (±SEM) of *Tgfb1*, *Tgfbr2 and Tgfbr1* of sorted fetal liver monocytes (Liv Mo) at E15.5, fetal lung monocytes (Lg Mo), preAMs and Liv Mos at E18.5, and adult AMs and BM monocytes (BM Mo). N = 3. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant (one-way anova).

Next, we analyzed $Lyz2^{Cre}Tgfbr2^{fl/fl}$ (homozygous for Cre) embryos at E18.5. Fetal monocytes in the developing lung were unaffected in $Lyz2^{Cre}Tgfbr2^{fl/fl}$ mice compared to control littermates (Figure 15A and Figure 16A). However, a drastic decrease in preAMs ($Ly6C^{lo}CD64^{hi}$) was noted. Within this remaining preAM population, a second $CD11b^{hi}$ population emerged in the $Lyz2^{Cre}Tgfbr2^{fl/fl}$ lungs (Figure 15A). This atypical $CD11b^{hi}$ population arose as a result of the loss of *Tgfbr2* signaling as verified by RT-PCR (Figure 15B). In contrast, the $CD11b^{lo}$ preAM population observed in $Lyz2^{Cre}Tgfbr2^{fl/fl}$ mice, which resembles normal preAMs, were not targeted and had an undisrupted *Tgfbr2* locus (Figure 15B). For complete targeting of all hematopoietic cells in the embryo, we again used the $Vav1^{Cre}Tgfbr2^{fl/fl}$ mice, in which we found AM development completely abolished at E18.5 (Figure 15C). *Tgfbr2* deletion on fetal liver monocytes, which was confirmed by RT-PCR, did however not hamper their differentiation into tissue macrophages other than the lung (such as liver, spleen, kidney, gut and dermis) (Figure 16A-B). In other words, TGF-βR signaling does not affect fetal liver monocyte development nor their ability to become tissue macrophages *per se*, but is highly specific to the normal developmental transition of fetal monocytes into AMs.

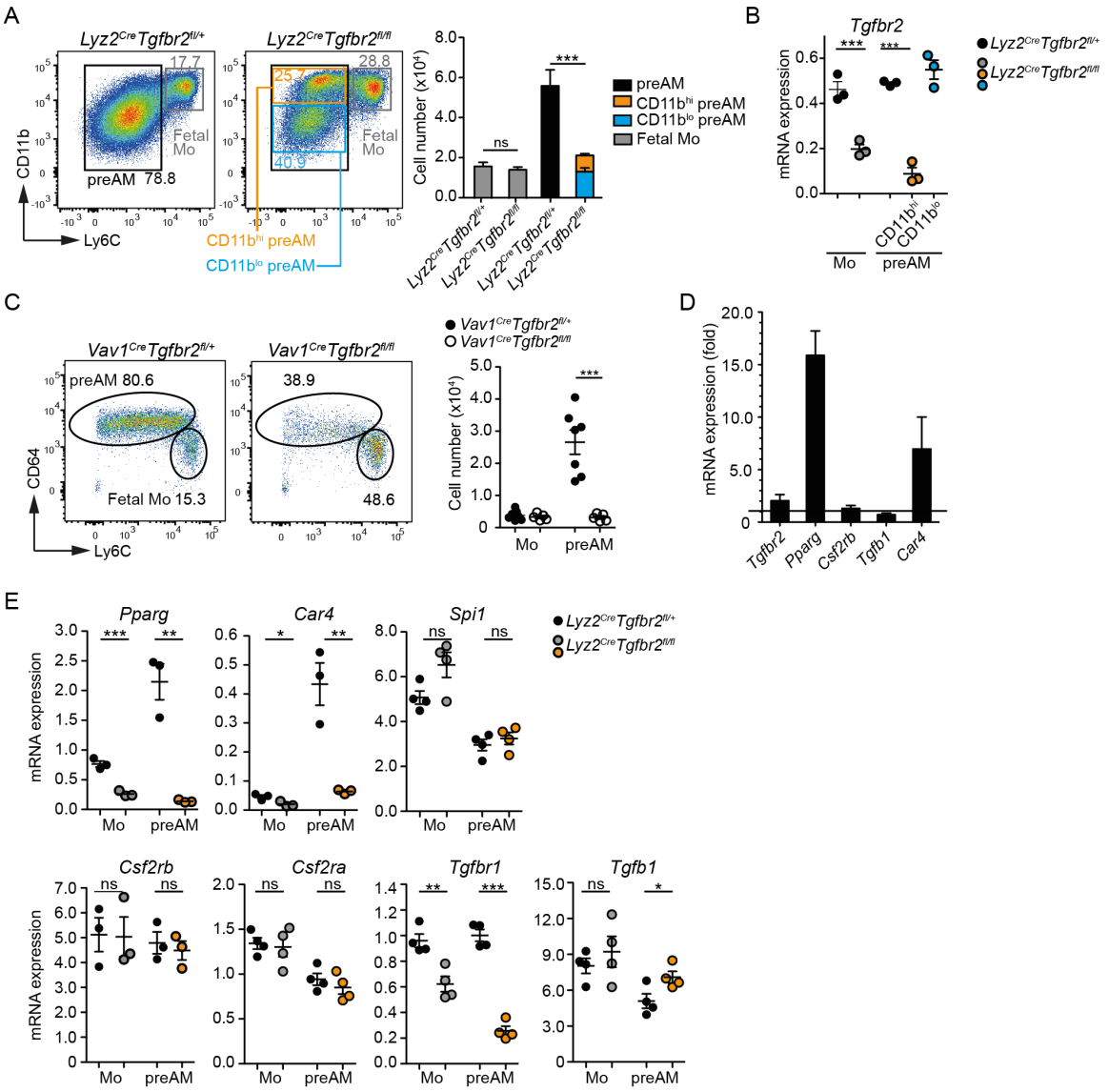

Figure 15. TGF-βR signaling is indispensable for the development of AMs during embryogenesis. **(A)** Representative flow cytometry plots (left) and quantification of total cell numbers (±SEM) (right) of fetal monocytes (fetal Mo, Ly6C^{hi}CD11b^{hi}) and preAMs in *Lyz2^{Cre}Tgfbr2$^{fl/fl}$* and *Lyz2^{Cre}Tgfbr2$^{fl/+}$* (homozygous for *Lyz2^{Cre}*) embryos at E18.5 (pre-gated on CD45^{+}Ly6G^{-}MHCII^{-}F4/80^{lo}CD11b^{+}CD64^{+} cells). N = 13, 4 independent experiments. **(B)** Relative mRNA expression (±SEM) of *Tgfbr2* from sorted fetal monocytes and preAMs as shown in (A) normalized to *Pol2*. N ≥ 3, each from 2-4 pooled mice. **(C)** Flow cytometry analysis of fetal lung of *Vav1^{Cre}Tgfbr2$^{fl/fl}$* and *Tgfbr2$^{fl/fl}$* mice at E18.5. Total cell numbers (±SEM) are quantified on the right. N ≥ 5, from 2-3 independent experiments. **(D)** Fold change of relative mRNA expression of *Tgfbr2, Pparg*, *Csf2rb*, *Tgfb1* and *Car4* from E15.5 fetal liver monocytes cultured *in vitro* with TGF-β1 or with anti-TGF-β1 blocking antibodies, normalized to *Pol2*. N = 3 from 3 independent experiments. **(E)** Relative mRNA expression levels of *Pparg, Car4, Spi1, Csf2rb, Csf2ra, Tgfbr1 and Tgfb1* from sorted fetal monocytes and preAMs as described in (A-B), normalized to *Pol2*. N = 3, each from 2-4 pooled mice. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant (unpaired Student's t test).

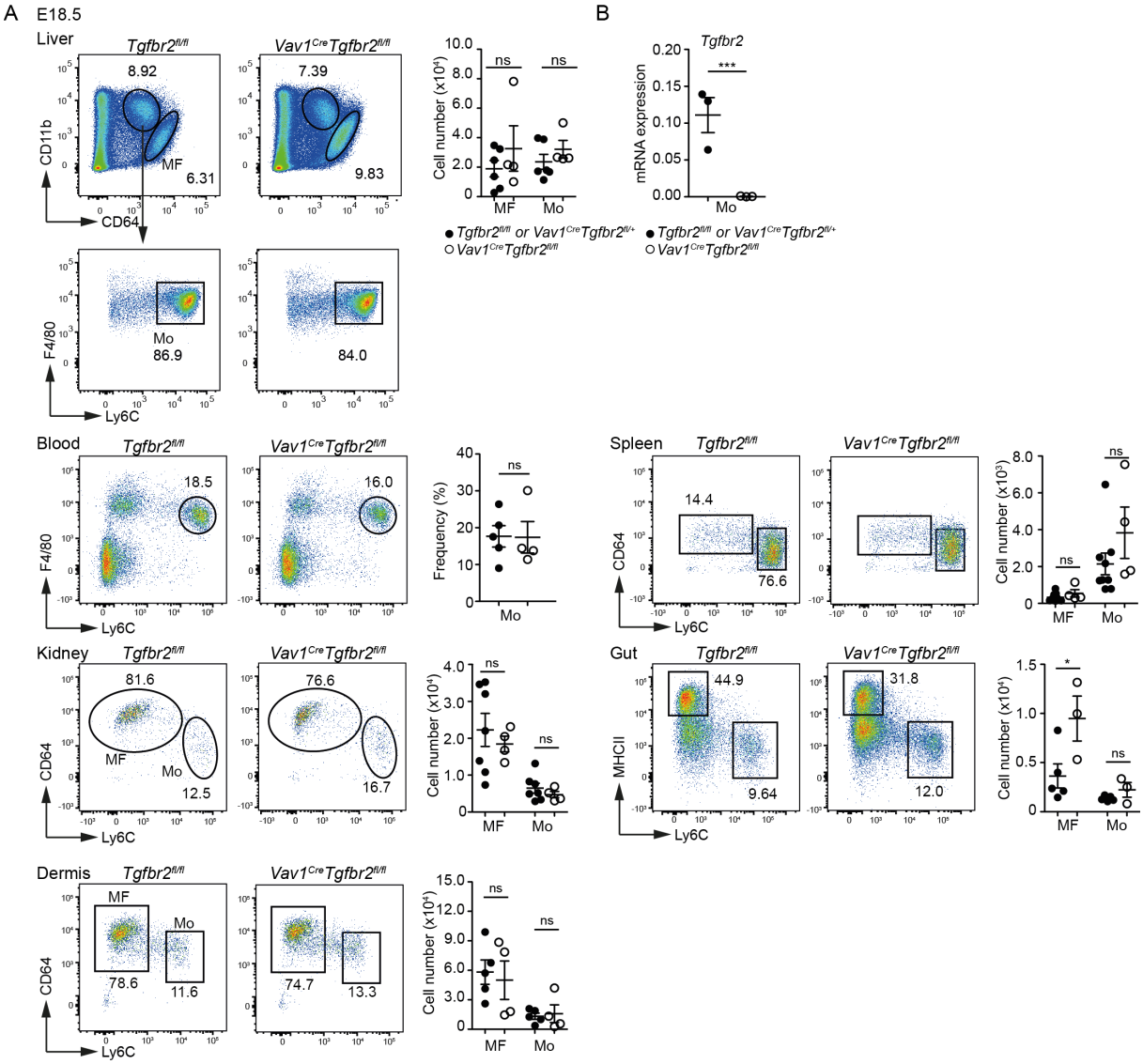

Figure 16. TGF-β is not essential for the development of other macrophages. (A) Representative flow cytometry plots (left) and quantification of total cell numbers (±SEM) (right) of fetal liver monocytes (Mo, CD11b^{+}Ly6^{hi}CD64^{mid}) and macrophages (MF, CD11b^{+}CD64^{hi}), blood Mo, and Mo and MF in kidney, gut, spleen and dermis in *Vav1^{cre}Tgfbr2$^{fl/fl}$* and *Tgfbr2$^{fl/fl}$* embryos at E18.5 (pre-gated on CD45^{+}Ly6G^{-}MHCII^{-}Siglec-F^{-} cells). N = 4-6 from 3 independent experiments. **(B)** Relative mRNA expression levels (±SEM) of *Tgfbr2* of sorted fetal liver monocytes as in (A) normalized to *Pol2*. N ≥ 3, each from 1-2 mice. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant (unpaired Student's t test).

To investigate how TGF-β instructs AM development, we sorted fetal monocytes from WT embryonic livers at E15.5 and cultured them with GM-CSF in the presence or absence of TGF-β. As readout for AM differentiation, we analyzed the expression of *Pparg* and *Car4*, both AM signature genes. While GM-CSF served as a survival factor (data not shown), the addition of TGF-β induced the expression of *Pparg* and *Car4* (Figure 15D). Expression of *Csf2rb*, *Tgfbr2* and *Tgfb1* was not affected by TGF-β. Moreover, when we replaced GM-CSF with M-CSF to sustain the cell culture,

addition of TGF-β again induced *Pparg* expression (data not shown). This finding suggests that TGF-β stimulation *in vitro* induces gene expression in fetal monocytes that allow AM differentiation (*Pparg*) or are related to AM identity (*Car4*).

Conversely, we also analyzed the expression of these genes *in vivo* in *Tgfbr2*-deficient preAMs (CD11b^{hi}) in *Lyz2^{Cre}Tgfbr2$^{fl/fl}$* embryos. We found that *Pparg* and *Car4* were weakly expressed in *Tgfbr2*-deficient preAMs in contrast to control preAMs (Figure 15E). The expression of the pioneer transcription factor for the myeloid lineage PU.1 (encoded by *Spi1*) was not affected by the disruption of TGF-βR signaling. On the other hand, expression of *Tgfbr1* was reduced in *Tgfbr2$^{-/-}$* preAMs while *Tgfb1* was not affected. We next analyzed whether TGF-βR signaling regulates GM-CSFR. We found that the expression of *Csf2ra* and *Csf2rb* was unaltered upon deletion of TGF-βR in preAMs (Figure 15E). Equally, analysis of fetal lung monocytes from *Csf2rb$^{-/-}$* embryos, in which their differentiation into preAMs is disrupted, revealed that expression levels of *Tgfbr2, Tgfbr1* and *Tgfb1* were also unchanged (Figure 17A). Taken together, TGF-βR signaling does not impact on GM-CSFR expression in preAMs, or vice versa, but both pathways instruct the expression of *Pparg,* which is critical for AM differentiation.

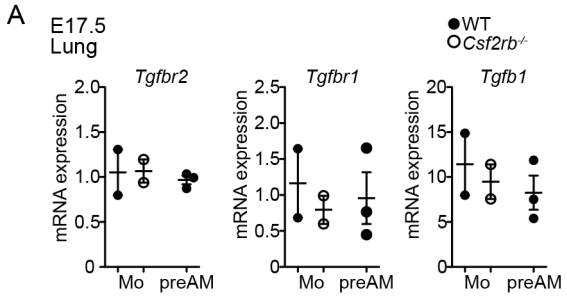

Figure 17. GM-CSFR signaling has no impact on TGF-βRs and ligand expression in preAMs (A) Relative mRNA expression of *Tgfbr2, Tgfbr1 and Tgfb1* from fetal liver monocytes of *Csf2rb$^{-/-}$* and WT mice and preAMs of WT mice at E17.5. N = 2-3, each from 2 mice. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant (unpaired Student's t test).

6.6 TGF-β Instructs AM Differentiation and Signature Gene Expression

CD11c expression on AMs commences within the first 3 days postnatally. We observed that *Itgax*^{Cre}*Tgfbr2*$^{fl/fl}$ mice at P3 showed reduced, yet detectable numbers of AMs while at P7, AMs were already absent indicating that TGF-βR signaling is also critical for the maturation of AMs after birth (Figure 18A and data not shown). Thus, to analyze the impact of TGF-β on the entire transcriptome of AMs, we performed RNA-sequencing (RNA-seq) on AMs deficient of *Tgfbr2* in *Tgfbr2*$^{fl/fl}$ mice at P3 but still present at cell numbers that allowed sorting. Reduced expression of approximately 60% of *Tgfbr2* (exon 4) was observed in *Itgax*^{Cre}*Tgfbr2*$^{fl/fl}$ AMs (Figure 9B). This incomplete deletion reflects the variable differentiation stages of AMs as not all have undergone genetic recombination at this time point. Nevertheless, we found that of a total of 10,825 expressed genes, 543 genes were differently expressed ($P < 0.001$) (Figure 18C). To verify a specific targeting of the TGF-βR pathway, we analyzed expression of genes previously described to be associated with TGF-βR signaling (Figure 18D-E)[159,160]. For example, TGF-β-induced genes, *Hpgd, Serpine1* and *Gcnt2* were down-regulated in *Tgfbr2*-deficient AMs[161–164]. Also, genes involved in latent TGF-β activation such as *Thbs1* and *Itgb5* were expressed at low levels compared to control AMs[158,165]. Negative regulators of the TGF-β signaling pathway such as *Smurf2, Ski* and *Skil*, whose expression positively correlates with TGF-β stimulation, were lower expressed in *Tgfbr2*$^{-/-}$ AMs[166,167].

Furthermore, we also found several AM signature genes to be down-regulated in *Tgfbr2*-deficient AMs[53]. Among those were for example *Scgb1a1*, *Epcam* and *Cyp4f18* (Figure 18F), some of which were among the top down-regulated genes (Figure 18G). Genes highly expressed in monocytes but not in mature macrophages were up-regulated in *Tgfbr2*-deficient AMs[53]. For example, *Ndrg1* and *Irf7*, which are both implicated in the development of macrophages *in vitro*[168,169]. Conversely, *Adamdec1,* induced in macrophages upon their differentiation from monocytes[170], was lower expressed in *Tgfbr2*-deficient AMs.

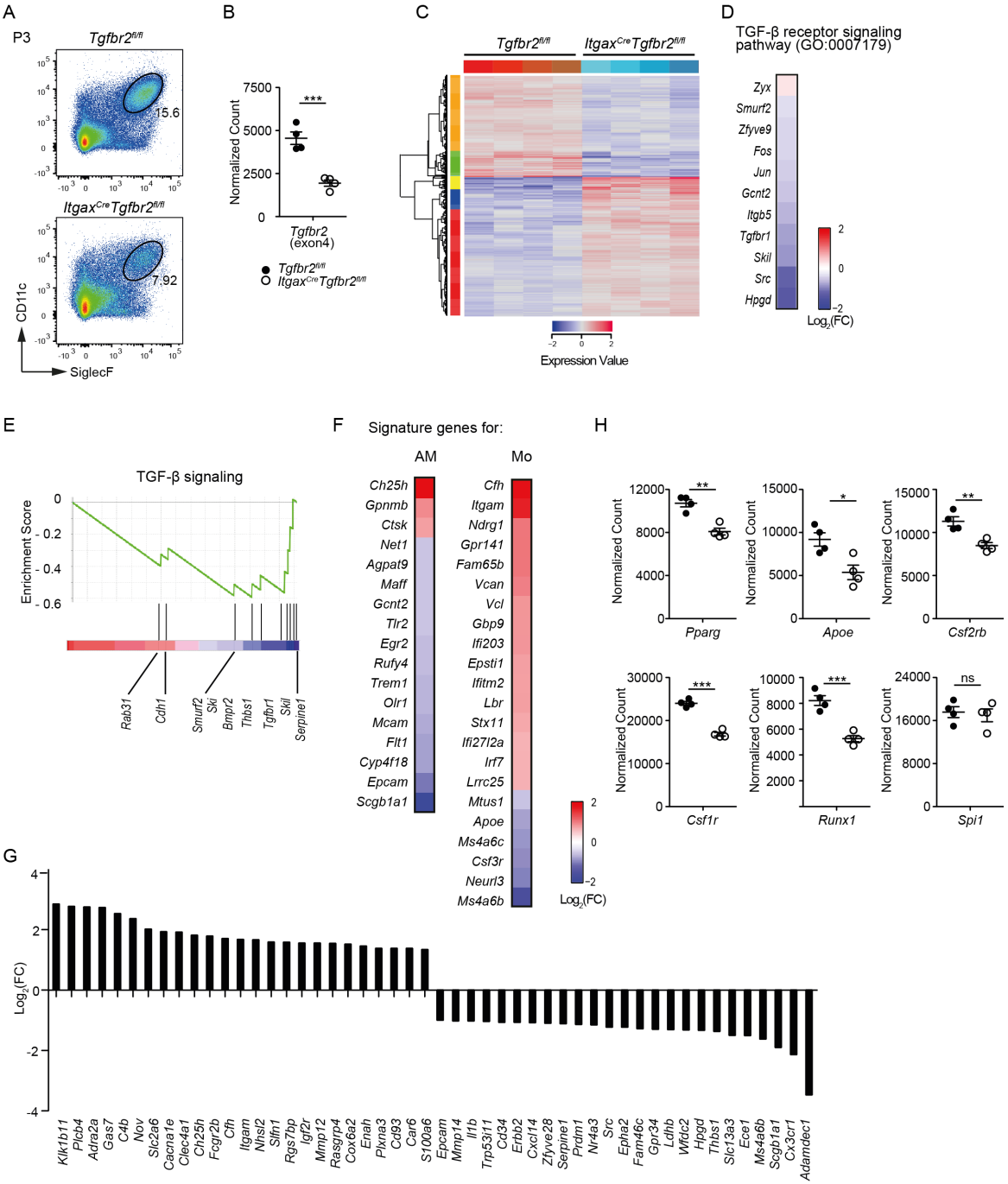

Figure 18. Gene expression profiles of *Tgfbr2*-deficient AMs. (A) Flow cytometry plots show the percentage of AMs (Siglec-F^{+}CD11c^{+}), pre-gated on CD45^{+} cells) of *Itgax*^{Cre}*Tgfbr2*$^{fl/fl}$ mice and control littermates (*Tgfbr2*$^{fl/fl}$ or *Itgax*^{Cre}*Tgfbr2*$^{fl/+}$) at P3. **(B-H)** AMs were sorted from *Itgax*^{Cre}*Tgfbr2*$^{fl/fl}$ mice and control littermates at P3 as in (A) for NGS. (See also gating strategy for NGS in Figure S1C). N = 4, each from 2-4 mice. **(B)** Normalized expression counts of Exon 4 of *Tgfbr2*. **(C)** Heat map of expression values of genes expressed differentially in *Itgax*^{Cre}*Tgfbr2*$^{fl/fl}$ or *Tgfbr2*$^{fl/fl}$ AMs. (Significant p value < 0.001, FDR = 0.020). **(D)** Heat map showing differentially expressed genes belonging to the TGF-βR signaling pathway (GO:0007179, $\log_{10}$P = -5.25, fold change (FC)). **(E)** GSEA of genes expressed differentially in *Itgax*^{Cre}*Tgfbr2*$^{fl/fl}$ compared to *Tgfbr2*$^{fl/fl}$ AMs. Enrichment plot for 'TGF-β signaling'. (P = 0.0045, FDR= 0.034). **(F)** Differentially expressed AM and monocyte signature genes[53] in *Itgax*^{Cre}*Tgfbr2*$^{fl/fl}$ and *Tgfbr2*$^{fl/fl}$ AMs ($\log_2$ (FC) > 0.5) **(G)** Fold change in expression of the 25 most significantly up- or down-regulated genes in AMs from *Itgax*^{Cre}*Tgfbr2*$^{fl/fl}$ mice compared to *Tgfbr2*$^{fl/fl}$ mice. **(H)** Normalized expression counts of *Pparg*. *Runx1*, *Csf1r*, *Csf2rb* and *Spi1*. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant (unpaired Student's t test).

Lipid metabolism in AMs is critical for surfactant catabolism. Consistent with the mRNA expression data of preAMs (Figure 15E), in the absence of *Tgfbr2* we observed a reduction in *Pparg,* the master transcription factor involved in lipid metabolism[171] (Figure 18H). Other genes such as *Apoe*, mediating cholesterol efflux and preventing foam cell formation, and *Olr1*, a scavenger receptor mediating oxLDL update, were also down-regulated in AMs lacking *Tgfbr2*[94,172] (Figure 18F, H). Furthermore, the expression of *Csf1r* and *Csf2rb* were both reduced upon deletion of *Tgfbr2* in AMs. *Runx1*, a transcription factor highly expressed in hematopoietic progenitor cells[173,174], was also decreased in the absence of *Tgfbr2*. On the other hand, *Spi1* was not affected (Figure 18H). Overall, these data suggest that TGF-βR signaling leads to transcriptional changes in AM differentiation, maturation and signature.

6.7 PPAR-γ Agonist can not Rescue *Tgfbr2*-deficiency Induced AM Loss

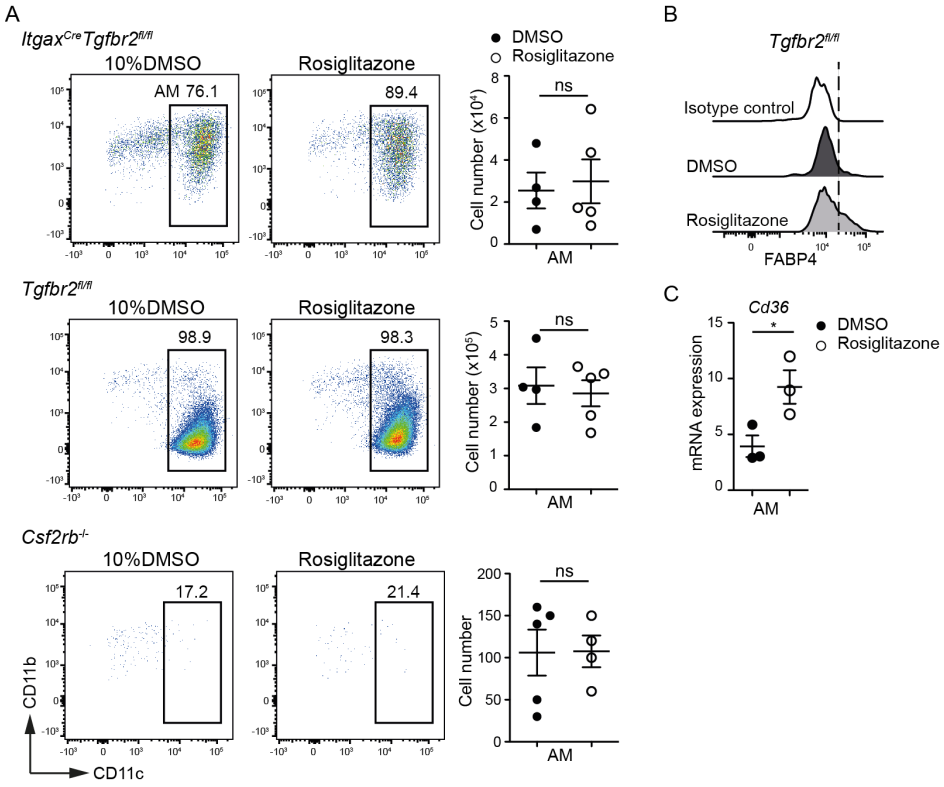

Figure 19. Rosiglitazone could not rescue AM loss in *Tgfbr2*-deficient mice. (A) Neonatal *Itgax*^{Cre}*Tgfbr2*$^{fl/fl}$, *Tgfbr2*$^{fl/fl}$ and *Csf2rb*$^{-/-}$ mice (P2-P4) were treated with 10 µg Rosiglitazone in 10% DMSO or 10% DMSO every day for 12 days i.n. and analyzed one day after the last treatment (A-C). **(A)** Flow cytometry of AMs (CD11c^{+}, pre-gated on CD45^{+}Ly6C^{-}Ly6G^{-} F4/80^{+}CD64^{+} SiglecF^{+} live cells) from the lung (left) and quantification of total cell numbers of AMs (right). **(B)** Flow cytometry analysis of FABP4 expression by AMs as shown in (A) from *Tgfbr2*$^{fl/fl}$ mice treated with DMSO or Rosiglitazone. N = 3, one representative histogram is shown. **(C)** qRT-PCR of *Cd36* mRNA from AMs sorted from *Tgfbr2*$^{fl/fl}$ mice either treated with DMSO or Rosiglitazone, normalized to *Pol2* expression, N = 3. ±SEM is shown. *p < 0.05, ns, not significant (unpaired Student's t test).

Previously, we have concluded that *Pparg* is regulated by TGF-βR signaling in both preAMs and AMs. Whether TGF-βR deficiency mediated PPAR-γ loss fully explains the phenotype of AMs we observed is not clear. To address this question, we treated *Itgax*^{Cre}*Tgfbr2*$^{fl/fl}$ neonates with PPAR-γ agonist rosiglitazone intranasally (i.n.) for 12 days. This did not however significantly influence total AM numbers (Figure 19A). Functionality of the compound was confirmed by the anticipated upregulation of PPAR-γ targets[175,176] *CD36* and FABP4 on WT AMs (Figure 19B-C). Moreover and importantly, the link between GM-CSF and PPAR-γ is widely held as solid. However, treating *Csf2*$^{-/-}$ [60] or *Csf2rb*$^{-/-}$ mice (Figure 19A) with rosiglitazone also failed to restore AM numbers. Thus, these data illustrate that administration of rosiglitazone is insufficient to overcome the loss of AMs driven by ablation of *Tgfbr2*. Taken

together, independent of the pathway controlling *Pparg* (*Tgfbr2* or *Csf2rb*), loss of *Pparg* and AMs cannot be restored with rosiglitazone, indicating that more profound changes than merely PPAR-γ loss induced transcription disruption in AMs occur upon *Tgfbr2* or *Csf2rb* deletion.

6.8 Flu-induced AM Loss is not Associated with the Reduced Expression of Pulmonary TGF-β and TGF-βRs.

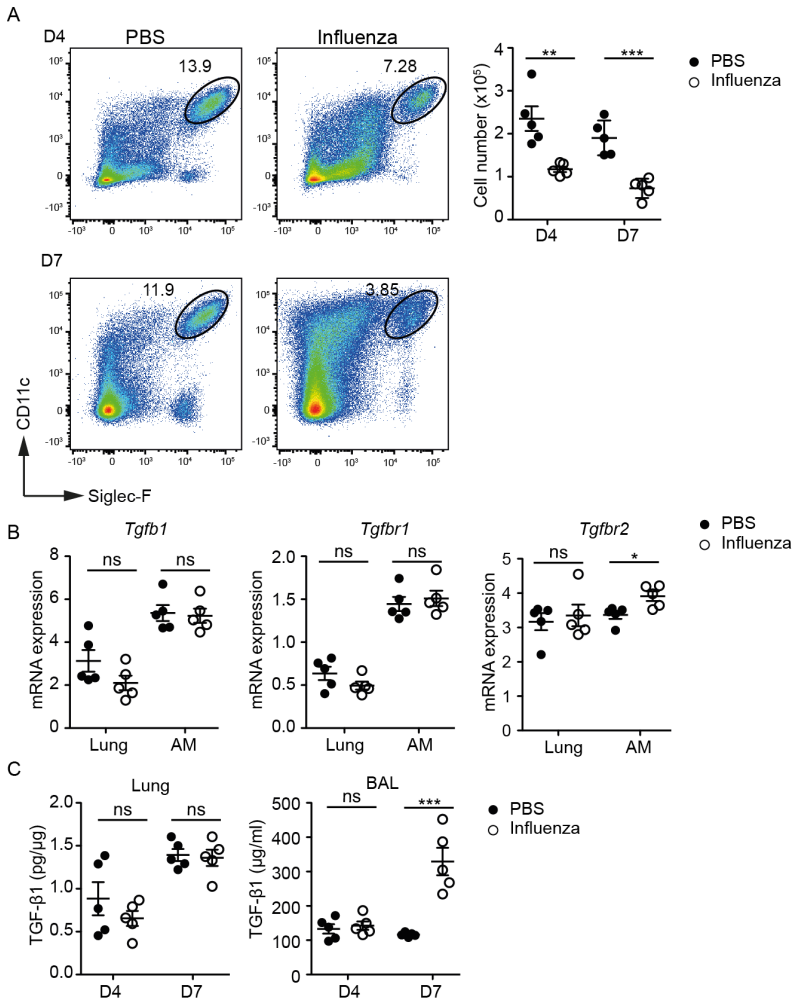

Figure 20. The expression of TGF-βRs and ligand post influenza virus infection. (A-C) WT mice were administrated with either 5000 PFU influenza virus (PR8) or PBS i.n. and analyzed at D4 or D7 post infection. **(A)** The number of AMs (Siglec-F^{+}CD11c^{+}, pre-gated on CD45^{+} cells) at D4 and D7 was quantified by flow cytometry analysis. **(B)** qRT-PCR of *Tgfb1*, *Tgfbr1* and *Tgfbr2* mRNA from sorted AMs or total lung tissue at D4, normalized to *Pol2* expression. **(C)** Elisa of total TGF-β1 from lung tissue or from BAL at D4 and D7. N = 5. ±SEM, *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant (unpaired Student's t test).

The macrophage disappearance reaction is a common observation in the lung immunology field[177,178]. To address whether inflammation induced loss of AMs relates to TGF-βR signaling, WT mice were infected i.n. with influenza virus PR8, which expectedly leads to a decrease of AMs as previously described[58] (Figure 20A). However, influenza virus infection did not significantly alter *Tgfb1* expression in AMs (Figure 20B). Thus, autocrine TGF-β signaling is still intact, which does not support reduced TGF-β signaling as a mechanism of AM reduction. Also, at a later

point in time, when AMs are already reduced, we found a significant increase of TGF-β in the BAL but not in the lung tissue (Figure 20C). This increase in TGF-β is indicative of a response to inflammation as this commonly occurs across multiple inflammatory conditions. Taken together, it indicates that lack of TGF-β and its receptors is not the reason for the loss of AM in during influenza infection.

7 Discussion

7.1 TGF-β is Essential for the Differentiation and Homeostasis of AMs.

The development and maintenance of most macrophages is dependent on CSF-1R signaling. AMs, on the other hand, are much less affected by the absence of CSF-1R signaling than other tissue resident macrophages. This correlates with its low CSF-1R expression levels compared to other tissue macrophage populations[53]. Conversely, AMs, like LCs and microglia, express high levels of TGF-βRs in the steady state. Here we utilized different Cre/LoxP mouse lines with *Tgfbr2*-specific deletion to study the role of TGF-βR signaling in the development and maintenance of AMs and other macrophage populations.

In $Itgax^{Cre}Tgfbr2^{fl/fl}$, $Lyz2^{Cre}Tgfbr2^{fl/fl}$, and $Vav1^{Cre}Tgfbr2^{fl/fl}$ mice where AMs lack TGF-βR2 expression, the lungs are devoid of AMs. Although associated with various degree of tissue inflammation as described previously[145,150], other myeloid cell populations that are targeted in these mice, for example pulmonary DCs in $Itgax^{Cre}Tgfbr2^{fl/fl}$ mice and macrophages from kidney and small intestines in $Vav1^{Cre}Tgfbr2^{fl/fl}$ mice are not significantly altered in number. Exceptions are LCs, known to be deficient in the absence of TGF-βR signaling, that were found to be depleted and red pulp macrophages that were also reduced. We demonstrated that AMs are unique in their complete dependency on TGF-βR signaling during development and high sensitivity to the lack of TGF-βR signaling. Consistent with this, Tamoxifen-induced depletion of TGF-βR2 in adult $R26^{CreER}Tgfbr2^{fl/fl}$ mice also led to a drastic reduction of AMs in number, indicating that TGF-βR signaling is constantly required by AMs for their homeostasis after birth.

The impact on cellular events following the disruption of TGF-βR signaling in adult AMs has not been identified yet. This is not likely due to impaired cell division since the turnover rate of AMs is much slower than the speed of AM loss as we observed here[60]. Meanwhile, we did not observe altered rate in cell death in the *in vitro* AM culture (data not shown). It has been suggested by many studies that AM identity and function are highly dependent on its contact with lung epithelia[73,97,98], leading to the hypothesis that loss of TGF-βR signaling affects AM adhesion with epithelial cells. In

RNA-sequencing experiment assessing the transcriptomic programs of *Tgfbr2*-deficient AMs and WT AMs, we identified multiple genes involved in cell attachment and migration highly downregulated in Tgfbr2-deficient mice. Nevertheless, in physiological analyses using immunofluorescent imaging and flow cytometry, we could not identify strong changes in AM adhesion to epithelia in the absence of TGF-βR signaling during the processes of AM loss in tomaxifen administrated $R26^{CreER}Tgfbr2^{fl/fl}$ mice. Technically, to better observe and define attached AMs and detached AMs with electron microscopy should help us to study the direct changes in cellular behaviors of AMs upon *Tgfbr2*-deletion.

During embryogenesis, we further characterized that preAMs at E18.5 are present as $CD11b^{lo}Ly6^{lo}CD64^{+}F4/80^{+}SiglecF^{-}CD11c^{-}$ population whereas their precursors, the fetal monocytes, are $CD11b^{hi}$ $Ly6C^{hi}$ similarly to data from previous findings[60,94]. In mice lacking TGF-βR2 at various time points during embryogenesis, we found a differentiation disruption and arrest specific to preAM, but neither to other tissue resident macrophages nor at fetal monocyte level. This demonstrates that TGF-βR signaling is specifically required for the differentiation of preAM from fetal monocyte, whereas the survival and migration of fetal monocyte itself is not TGF-βR signaling dependent. Interestingly, while $Vav1^{Cre}Tgfbr2^{fl/fl}$ mice had a complete loss of preAM population, a remained preAM population was found in $Lyz2^{Cre}Tgfbr2^{fl/fl}$ mice, in which a second *Tgfbr2*-deficient $CD11b^{hi}$ population emerged. It can be explained by the insufficient and late targeting in $Lyz2^{Cre}$ where $Lyz2^{Cre}$ is only initiated at pulmonary fetal monocyte stage rather than fetal liver monocyte stage. This insufficient targeting on the other hand leaves a window for exploring the downstream effects after TGF-βR2 depletion during development.

Although a wide range of cells in the lung can secrete TGF-β, here we show that an autocrine source of TGF-β is essential for AMs maintenance in adult. The source of TGF-β during embryonic AM development is still unclear and demands further investigations. In fact, AM itself produces more abundant TGF-β compared to epithelial cell, the only cell type that has direct contact with AM in the steady state. At the same time, integrin αvβ6 expressed by lung epithelial cells has been shown to play a crucial role in TGF-β activation[107]. Our findings add to this current model that

the epithelial cells offer avβ6 to activate AM derived inactive TGF-β and in turn sustain an active TGF-βR signaling for AMs.

7.2 Regulatory Networks of AM Development

Tissue-resident macrophages are functionally and phenotypically distinct populations located in different organs. The commitment to macrophage relies on pioneer transcription factor PU.1 and other macrophage lineage-defining factors. On top of that, tissue-specific transcription factors and induced epigenetic landscapes define the unique identity for each tissue-resident macrophage population. However, the understanding of how local mediators contribute to the establishment of such diverse gene expression patterns in different tissues is still limited. Here, in the lung, we identified TGF-β as a critical factor that regulates expression of genes associated with AM differentiation and fate.

TGF-β was previously shown to be critical for the development of LCs and was also implicated in the formation of microglia[93,148,179,180]. Our work demonstrated that TGF-β is also a prerequisite for the embryonic development of AMs and their maintenance throughout life. In LCs, TGF-βR signaling was shown to induce the expression of transcription factors Id2 and Runx3, both essential for LC commitment[144,181]. In microglia, TGF-β leads to the expression of the transcriptional regulator Sall1, which is highly expressed in microglia but not in other tissue macrophages[148,157]. Here we showed that TGF-βR signaling in fetal monocytes leads to the upregulation of PPAR-γ, a key transcription factor for the genesis of AMs. A few studies linked TGF-β to PPAR-γ, however, mainly demonstrating a negative regulation. It has been shown, for example, that PPAR-γ antagonizes TGF-β pathway or that TGF-β signaling negatively modulates the transcriptional activity of *Pparg*[182,183]. Another report has demonstrated the phosphorylation of PPAR-γ upon TGF-βR signaling[184]. Here, our data suggest that PPAR-γ is directly induced by the TGF-βR signaling pathway. Alternatively, the upregulation of PPAR-γ could also be a consequence of TGF-β-induced AM differentiation, which correlates with *Pparg* expression. Altogether, this suggests that TGF-βR signaling in macrophages is linked to macrophage 'signature' genes encoding transcription factors specific for either their development (PPAR-γ for AMs, Id2 and Runx3 for LCs) or their fate (Sall1 for microglia). We demonstrated that the development of other tissue macrophages than the aforementioned ones do not depend on TGF-βR signaling.

Among tissue resident macrophages, the genesis of AMs is also unique in its high dependency on GM-CSFR signaling. GM-CSFR signaling has been shown to be essential for the differentiation of fetal monocytes into immature AMs perinatally and for the full maturation of AMs postnatally but not for the accumulation of fetal monocytes in the developing lung[60,94]. Our data demonstrated that, in parallel to GM-CSFR signaling, TGF-βR signaling also functions at the stage of preAM differentiation and maturation but not at fetal monocyte level. TGF-βR and GM-CSFR signaling converge to induce the expression of PPAR-γ, a key factor for the transcriptional program specific to AMs[94]. The requirement for two individual cytokines for macrophage development is also reminiscent of LCs, which are dependent on IL-34 and TGF-β[91,93].

Despite the equal necessity for the genesis of AMs, GM-CSFR and TGF-βR pathways induce differential transcriptional behaviors that function complementarily in AM development and maintenance. GM-CSF mediated AM differentiation and homeostasis is largely through the pioneer transcription factor PU.1[114] that functions at the common macrophage lineage commitment level. PU.1 expression restores the functionality of GM-CSFR deficient AMs and GM-CSF controls *Spi1* (gene encoding PU.1) transcription[185–187]. In contrast, TGF-βR has no impact on *Spi1* transcription at both embryonic and neonatal stages of AM development. Together, it suggests that TGF-β is involved in the induction of AM identity, while GM-SCF might serve as a counterpart of CSF-1 in the lung to maintain PU.1 expression [188].

GM-CSF is secreted by epithelial cells and acts in a paracrine manner on AMs[175], while AMs are dependent on autocrine TGF-β in adulthood. During development, the fetal lung expresses higher levels of GM-CSF than other organs including heart, spleen, brain, kidney and liver[175], whereas TGF-β is present in both the fetal liver and lung at similar level (data not shown). The receptors for TGF-β are upregulated after fetal monocytes migrate from liver to the lung and are further increased when they become mature AMs after birth. It will be interesting to analyze factors that control TGF-βR expression during AM development.

The crosstalk between GM-CSFR and TGF-βR signaling in AMs remains to be discovered. We did not observe an impact of GM-CSFR deletion directly on the expression level of *Tgfbrs* and *Tgfb1* and *vice versa*, *Tgfbr2*-deficient AMs had

unaltered *Csf2r* expression at embryonic stage. At P3, *Tgfbr2*-deficient AMs, however, showed decreased *Csf2r* expression. At the intracellular signaling level, TGF-βR and GM-CSFR signaling networks overlap in many branches, such as the MAPK/ERK, PI3K/AKT and SRC pathways[113,118]. Normal activation states and the dynamics of these pathways might depend on both TGF-βR and GM-CSFR signaling that coordinately regulates downstream transcriptional events and determine cell fate. Lacking one of the signaling activities might alter the signaling events of the other, resulting in the disturbance of the entire cellular behaviors.

As the common target of TGF-βR signaling and GM-CSFR signaling, PPAR-γ itself as the only master transcription factor for the genesis of AMs is under debate. It is only until recently when researchers characterize PPAR-γ as an AM-specific transcription factor that people treat the high expression of PPAR-γ as equivalent to the AM identity[85,108,175]. It is true that as a master for lipid metabolism PPAR-γ undertakes important and specific function for AMs[175,189]. Indeed, no data support that PPAR-γ alone could rescue the loss of tonic signaling activity either with what we have shown here the loss of TGF-βR2 or in literature the lack of GM-CSF[60]. These studies mainly use PPAR-γ agonists. A true restoration of *Pparg* mRNA should better reflect to what extent could PPAR-γ overcome the loss of tonic cytokines. Similarly, experiments with overexpression of *Pparg* in other tissue-resident macrophages are missing. In summary of current findings, AM identity should not be over simplified and limited to the presence of PPAR-γ.

We could not detect *Bach2* expression by preAMs at E17.5 (data not shown) nor by AMs at P3 based on sequencing reads, indicating that this transcription factor is not involved in AM genesis. For this reason, we did not continue the study of the impact of GM-CSFR and TGF-βR signaling on it. For future, unbiased characterization of the interplays between GM-CSFR and TGF-βR signaling in either *in vitro* stimulated AM or AM precursor culture or in fetal monocytes in the lung should help to identify key players in AM development.

7.3 Implications in Pulmonary Function and Diseases

It has been shown in both human and mice that deficiency in GM-CSF or its receptors leads to AM loss and further cause the disease PAP. Here, in mice lacking AMs due to deletion of TGF-βR, SP-D and total protein concentrations in the BAL were also found increased, indicating the onset of PAP. These results demonstrate that the development of PAP resulted not only from *Csf2* or *Csf2rb* deficiency, but also from the loss of tonic TGF-β, thereby supporting the importance of both pathways in forming and maintaining AMs. Of note, histomorphological changes manifest only in aged mice. This could so far not be addressed with the mice used here since strains lacking AMs succumb to multi-organ inflammation before they would develop PAP or conversely, AMs that escaped gene-targeting repopulated the empty niche. Altogether, our finding suggests that functional AMs are the key component in maintaining lung respiratory function. PAP is a direct consequence of AM loss, rather than solely based on one cytokine GM-CSF. In clinic, the contribution of TGF-βR signaling disruption to the AM loss and to the PAP development is worth further investigation.

Inflammation leads to cytoablation of tissue-resident macrophages with simultaneous increased monocytes, moDCs or monocyte derived macrophages[58]. Whether it correlates with the loss of TGF-β is not known. We did not observe altered *Tgfb1, Tgfbr1 or Tgfbr2* expression in AMs nor in the entire lung tissue in influenza virus (PR8)-infected WT mice, indicating that inflammation-induced macrophage loss is not a consequence of altered TGF-βR signaling. Nevertheless, the impact of infection on TGF-β extracellular activation and the actual signaling events after infection has not been studied here. In different infection and allergy models and at different time during the disease progression, the role of TGF-βR signaling and its impact on AMs and MdCs is complex and requires further study.

Our study focused on the steady state AMs and revealed that TGF-β is one of the factors that shape the proper developmental pathway and maintain the homeostatic function of AMs. By understanding the regulatory pathways in AM development, researchers could regenerate not only alveoli but healthy AMs in regenerative medicine and for transplantation purpose[190,191]. Study of the homeostatic niche leads

to the open questions: what are the mediators that determine AM functions in an inflammatory or diseased setting and how would monocyte-derived macrophages respond differently from the resident AMs? Understanding the inflamed and disease-related niche and comparing it to the required niche in the healthy tissue, we would find the answers to these questions and have efficient strategies to combat chronic diseases.

In line with this, our findings of TGF-β as an indispensible factor for homeostatic AMs add to the current knowledge of TGF-βR signaling in lung pathology. When choosing TGF-βR signaling as a therapeutic target for inflammation induced fibrosis and tissue remodeling of the lung, attention must be paid towards the influence of TGF-β on resident AMs. Traditionally, TGF-β is recognized as the major responsible factor for fibrosis and tissue remodeling in the chronic inflamed lung, for example in COPD and asthma patients and counteracting against TGF-βR signaling becomes an interesting therapeutic target. Recently, many studies suggest that mature AMs are the 'good' macrophages via suppressing inflammation, while MdCs are the ones exacerbating symptoms[103]. Thus, a potential protective role of TGF-βR should not be neglected in designing clinical treatment. In allergic asthma, pro-inflammatory macrophages expand and dominate in the BAL and after repeated allergen challenge, IL-4, IL-13, and IFN-γ direct or indirectly change macrophage functions[103]. How to reset the function of those macrophages is based on the homeostatic requirements of AM. Since we showed that TGF-βR signaling is indispensible for AMs in their self-maintenance, TGF-β could play a positive role in curing chronic lung disorder.

8 Conclusion

Whereas it is now firmly established that almost all tissue macrophages arise from an embryonic precursor and are self-maintained, the regulatory mechanism for their tissue-specific differentiation remains largely unknown. Current knowledge of mediators in AM development is limited to the cytokine GM-CSF and the AM-specific transcription factor PPAR-γ. Here we identified TGF-β as another crucial differentiation factor to control the formation of AMs in the developing lung and to maintain AM identity in an autocrine manner in the adult. The effect of TGF-βR signaling on AM development is specific, as the genesis of other macrophages does not require TGF-β receptor signaling. In parallel to GM-CSF, TGF-β regulates expression of proteins, including PPAR-γ, that is associated with AM differentiation and fate decision.

Taken together, we demonstrated that TGF-βR signaling promotes genesis, maturation and survival of AMs. These findings reveal an additional layer of complexity regarding the guidance cues, which govern the formation and diversity of tissue-resident macrophages. It contributes to defining healthy tissue microenvironment, based on which a better understanding of pulmonary disorders can be achieved in the future.

9 Methods

9.1 Experimental Animals.

C57BL/6 and C57BL/6-CD45.1 mice were purchased from Janvier Labs. *Itgax*^{Cre}, *Lyz2*^{Cre}, *Vav1*^{Cre}, *R26*^{CreER}, *Cx3cr1*^{CreER}, *Tgfbr2*$^{fl/fl}$, *Tgfb1*$^{fl/fl}$ and *Csf2rb*$^{-/-}$ (*Csf2rb*$^{LacZ/LacZ}$) were bred in-house[40,112,152,192–196].

Mice were bred and maintained in groups of 1-5 animals per cage in the specific pathogen-free facility of Laboratory Animal Services Center (LASC), University of Zurich. Unless otherwise stated, 6-12 week old littermates of both sexes were used. All animal experiments performed in this study were approved by the Swiss Veterinary Office.

9.2 Method Details

9.2.1 Genotyping

Biopsies (ear clips, toe tips or tail) were lysed for 1 h at 95°C in 200 µl 50mM NaOH. 30 µl 1M Tris-HCl pH 8.5 was added to neutralize the reaction. Samples were centrifuged at 12’000 rpm to pellet the tissue debris and the supernatant was used for DNA analysis.

For genotyping 1 µl of DNA template and 0.5 µl of each primer (10 µM, Thermo Fisher Scientific) was used in 1x Hot Fire Pol Blend master mix (Solis BioDyne). PCR reaction was performed on C1000 Thermal Cycler (BioRad) and PCR products were analyzed in a 1.5-2.0 % Agarose (Biocompare) gel containing GelRed (Biotium). The primers used are listed in below:

Mouse strain	Primer	Primer sequence	Ann. temp	Amplicons (bp)
Lyz2^{Cre}	1	CTT GGG CTG CCA GAA TTT CTC	62°C	wt: 350 mut: 700
	2	TTA CAG TCG GCC AGG CTG AC		
	3	GCATTGCTGTCACTTGGTCG		
Tgfb1^{fl}	1	AAG ACC TGG GTT GGA AGTG	60°C	wt: 216 mut: 277
	2	CCC AGA AAT GCC AGA TTA CG		
General *cre*	1	TCCAATTTACTGACCGTACAC	52°C	wt: none mut: 700
	2	CATCAGCTACACCAGAGACGGAAATC		
Vav1^{Cre}	1	CTA GGC CAC AGA ATT GAA AGA TCT	64°C	wt: 324 mut: 236
	2	CCCAAAGACCCACTCATTTGCAGC		
	3	TTGTCAAGACCGACCTGTCCG		
	4	GACGAGATCATCGCCGTCGGGCA		
Tgfbr2^{fl}	1	TAT GGA CTG GCT GCT TTT GTA TTC	58°C	wt: 422 mut: 575
	2	TGG GGA TAG AGG TAG AAA GAC ATA		

9.2.2 Bone Marrow Chimeras.

BM cells were flushed out from the bones (femur, tibia, humerus and hip). Cells were filtered through a 70 µm cell strainer, washed with PBS and red blood cells were lysed with lysis buffer (4.15 g NH_4Cl, 0.55 g $KHCO_3$, 0.185 g EDTA in 500ml ddH20). Mice were lethally irradiated (split dose, 2 x 550 Rad with a 24h interval) and were injected i.v. with at least 5×10^6 BM cells. Reconstituted mice were analyzed 6-10 weeks after the BM transplant.

9.2.3 Tamoxifen Treatment.

Tamoxifen (Sigma) was dissolved in corn oil at 25 mg/ml. 5 mg (200 µl) were administered via oral gavage (o.g).

9.2.4 Influenza Virus Infection

WT mice were anesthetized using isoflurane and administrated with either 5000 PFU influenza virus (PR8) in 30 µl PBS or 30 µl PBS alone i.n. and analyzed at D4 or D7

post infection. The body weight of mice was monitored every day post infection and a maximal of 10% weight loss was allowed during the whole experiment process.

9.2.5 Rosiglitazone Administration

Neonatal mice (P2-P4) were anesthetized using isoflurane and treated with 10 μg Rosiglitazone in 10% DMSO or 10% DMSO alone every day for 12 days i.n. and analyzed one day after the last treatment.

9.2.6 Cell Suspension Preparations.

Cell suspensions were prepared as previously described (Bogunovic et al., 2009; Greter et al., 2012a; Greter et al., 2012b). Briefly, Mice were sacrificed by CO2 inhalation and intracardially perfused with approximately 20 ml PBS. Ear (for dermis and epidermis), lung, spleen, kidney, small intestine and liver were carefully removed. Small intestine were first cleared off feces, cut open longitudinally, washed with HBSS (without Ca^{2+}/Mg^{2+}), plus 1.35mM EDTA and 2% FBS 3 times and incubated with HBSS (without Ca^{2+}/Mg^{2+}), plus 1.35mM EDTA, 2% FBS and 1mM DTT for 12 min. Lung, spleen, kidney, small intestine (cleaned) and liver were cut into small pieces, followed by a digestion in 0.4 mg/ml collagenase type IV (from Clostridium histolyticum) in HBSS (with Ca^{2+}/Mg^{2+}) supplemented with 10 % FCS for 45 min at 37 °C. Epidermis and dermis were separated after Dispase digestion (1.2 mg/ml in HBSS) for 1.5 h, cut into small pieces, incubated in 0.4 mg/ml collagenase type IV (Sigma-Aldrich) in HBSS with 10% FBS for 2 h. Digested tissues were passed through an 18 G syringe and filtered through a 70 μm cell strainer to obtain a homogeneous cell suspension. Liver cell suspensions were further enriched by a 30% Percoll gradient as previously described[91,109] and centrifuged at 1700 rpm for 30 min. After washing in PBS, red blood cells were lysed in 1 ml lysis buffer (4.15 g NH_4Cl, 0.55 g $KHCO_3$, 0.185 g EDTA in 500ml ddH20) and stained.

9.2.7 Flow Cytometry

Cell pellets were resuspended in the antibody mix in PBS and stained at 4 C° for 25 min. After washing with PBS, cell pellets were resuspended in FACS buffer (2 mM EDTA, 2% FCS in PBS) and analyzed. Flow cytometry was performed using an LSRII Fortessa (Becton Dickinson/BD Biosciences) and analyzed with FlowJo

software (Tree Star). Cell sorting was carried out using a FACSAria III. Fluorochrome-conjugated monoclonal antibodies (mAbs) specific for mouse I-A/I-E (clone M5/114.15.2), CD11b (clone M1/70), CD11c (clone N418), CD45 (clone 30-F11), CD45.1 (clone A20), CD45.2 (clone 104), CD115 (clone AFS98), Ly6C (clone AL-21 or HK1.4), Ly6G (clone 1A8), Siglec-F (clone E50-2440), CD3 (clone 17A2), CD45R (clone RA3-6B2), CD64 (clone X54-5/7.1), CD103 (clone 2E7), CD24 (clone M1/69), CD31 (clone 390), EpCam (clone G8.8) purchased either from BD Biosciences, eBioscience, or Biolegend. Anti-F4/80 (clone Cl:A3-1) mAb was purchased from AbD. Anti-mouse CD16/32 (Biolegend) was included in all the staining panel. Prior to detailed analysis cells were always gated on single, live cells, dead cells were excluded with the Fixable Viability Kit (Aqua or Near-IR staining, Biolegend).

9.2.8 t-SNE Display.

FCS files of single, live CD45+ cells (30,000 cells per sample) were exported from FlowJo X (Tree star) and uploaded to Cytobank (http://fluidigm.cytobank.org) for t-SNE visualization based on all surface markers in the staining panel, excluding CD45 and live/dead markers. Different key populations were gated manually based on t-SNE separation and overlayed back onto the t-SNE map. Subsequently, the marker expression levels for each population were extracted from Cytobank and visualized in R as a heatmap.

9.2.9 Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) was performed by flushing the lungs twice with 400 µl 0.5 mM EDTA/PBS using 22 G x 1.00 Insyte-W™ peripheral venous catheter (BD).

9.2.10 Oil Red O Staining

Cells derived from the BAL were spun onto slides precoated with 0.01% poly-L-lysine. After fixing for 10 min with 4% PFA, cells were washed with PBS and rinsed with 60% isopropanol, stained for 20 min with 0.3% Oil Red O (Sigma) in 60% isopropanol, followed by 2 min of destaining in 60% isopropanol and washing with PBS. Subsequently, cell nuclei were stained with hematoxylin for 2 min and rinsed

with tap water. Sections were mounted with immunoselect antifading mounting medium (Dianova).

9.2.11 H&E staining of Lung Tissue Sections

The lungs were perfused with PBS, removed, fixed in HOPEI for 72 hours, incubated in HOPEII/acetone for 2 hours, and followed by pure acetone incubation for 6 hours and paraffin incubation overnight. Lungs were embedded in paraffin and 5 µm sections were cut for H&E stainings. Briefly, sections were deparaffinized in Xylene for 5 min 3 times and rehydrated in 100% ethanol twice, 95% ethanol twice and 70% ethanol each for 3 min. After being rinsed with distilled water for 5 min, sections were stained in hematoxylin for 5 min, rinsed in running tap water for 1 min, and counterstained with Eosin for 1 min. Dehydration was done in serial rinsing twice in 95% ethanol and twice in 100% ethanol. After being cleared with Xylene for 3 min, sections were mounted in DPX mounting medium.

9.2.12 Immunofluorescence Staining of Lung Tissue Sections

Mice were euthanized with barbiturate and perfused with PBS. Lung was removed and fixed in 4% paraformaldehyde (Kantonsaptheke, Morphisto) for 48 h, followed by incubation in 30% sucrose (Sigma)/PBS solution for 24 - 48 h prior to embedding in OCT Cryo embedding medium (Medite) on dry ice. Cryo-sections were stored at -80°C. For immunohistochemistry, 20 µm sections were cut and washed once with PBS to remove OCT medium. Sections were blocked for 1 h at RT with 10% normal goat serum (NGS, Thermo Fisher Scientific) /0.5% Triton-x100 (Sigma)/PBS solution. Primary antibodies were diluted in 4% NGS /0.1% Triton-x100/PBS solution and incubated for 2 h at RT or overnight at 4°C. After washing with PBS, sections were incubated with secondary antibodies against the host IgG (Invitrogen) for 0.5-1 h at RT. Sections were washed again and covered with DAPI containing immunoselect antifading mounting medium (Dianova).

9.2.13 *In vitro* Culture of Fetal Monocytes

Fetal monocytes ($CD45^{+}Ly6G^{-}Ly6C^{+}CD11b^{+}CD64^{int}$) were sorted from fetal livers (E15.5) and cultured at 50,000 cells per well in 48-well plates in complete DMEM medium (PAN Biotech), plus 1 mM Sodium Pyruvate, 1x GlutaMAX (Gibco), 10%

FCS, 1% Pen/Strep (Gibco), 50 µM β-Mercaptoethanol (Gibco), 10 mM HEPES (Gibco) with 50 ng/ml GM-CSF (Biolegend). Cells were co-cultured with either 8.6 µg/ml mouse IgG1 isotype control (MOPC-21, BioXcell), 8.6 µg/ml anti-pan TGF-β1 antibody (clone: 1D11.16.8, BioXcell), or with 10 ng/ml hTGF-β1 (PeproTech) and 8.6 µg/ml mouse IgG1 isotype control for 24h. Cells were then processed for RNA isolation.

9.2.14 Quantitative RT-PCR (qRT-PCR).

Total RNA was isolated using RNeasy micro plus kit (Qiagen). cDNA was synthesized with M-MLV reverse transcriptase (Invitrogen) and qRT-PCR was performed on a C1000 Touch Thermo Cycler (Bio-Rad) using SYBR Green (Bio-Rad). Primers used are listed here:

Tgfbr2 forward: AAC GAC TTG ACC TGT TGC CTG T

Tgfbr2 reverse: CTT CCG GGG CCA TGT ATC TT

Tgfb1 forward: TGA CGT CAC TGG AGT TGT ACG G

Tgfb1 reverse: GGT TCA TGT CAT GGA TGG TG

Tgfbr1 forward: CAT TCA CCA CCG TGT GCC AAA TGA

Tgfbr1 reverse: ACC TGA TCC AGA CCC TGA TGT TGT

Pol2 forward: CTG GTC CTT CGA ATC CGC ATC

Pol2 reverse: GCT CGA TAC CCT GCA GGG TCA

Csf2ra forward: CTG CTC TTC TCC ACG CTA CTG

Csf2ra reverse: GAG ACT CGC CGG TGT ATC C

Csf2rb forward: GTG GAG CGA AGA GTA CAC TTG

Csf2rb reverse: CC AAA GCG AAG GAT CAG GAG

Pparg forward: GTG ATG GAA GAC CAC TCG CAT T

Pparg reverse: CCA TGA GGG AGT TAG AAG GTT C

Car4 forward: CTC CTT CTT GCT CTG CTG

Car4 reverse: GAC TGC TGA TTC TCC TTA

Spi1 forward: GAT GCA CGT CCT CGA TAC TC

Spi1 reverse: TCA TCT GAG CTC TGC TG GTG

Il1b forward: GAT CCA CAC TCT CCA GCT GCA

Il1b reverse: CAA CCA ACA AGT GAT ATT CTC CAT G

Tnf forward: CAT CTT CTC AAA ATT CGA GTG ACA A

Tnf reverse: TGG GAG TAG ACA AGG TAC AAC CC

Cd36 forward: TTA ATG GCA CAG ACG CAG CC

Cd36 reverse: TCA GAT CCG AAC ACA GCG TGA

9.2.15 ELISA

Tissue samples were lysed in ELISA lysis buffer (50 mM pH7.4 Tris, 5 mM EDTA, 150 mM NaCl, 1% NP-40 and proteinase inhibitors (Roche)). After homogenization, lysate were incubate at 4 C° for 20 mins and centrifuged at 4 C°, 12000 rpm for 10 min to remove debris. Protein concentrations of the supernatant were determined with a BCA protein assay kit (Pierce). ELISA for TGF-β1 and SP-D were performed according to the manufacturer's instructions (R&D).

9.2.16 Next Generation Sequencing

Total RNA was isolated from FACS-sorted AMs ($\geq 1.5 \times 10^5$ cells) using the RNeasy micro plus kit (Qiagen). 100 ng total RNA samples were poly-A selected and used for library preparation with TruSeq Stranded mRNA Sample Prep Kit (Illumina, Inc, California, USA). Next generation sequencing was performed by the Functional Genomics Center Zurich (FGCZ) using the HiSeq 2500 v4 System (Illumina). Bioinformatic analysis was performed using SUSHI platform developed by the

FGCZ. In short, Fastqc for quality control, STAR for mapping, DexSeq for exon counts, RSEM for transcript counts, and EdgeR for differentially expressed genes (DEG) were done with SUSHI. Gene ontology (GO) analysis by SUSHI and Gene Set Enrichment Analysis (GSEA) were performed on DEGs with $p < 0.001$.

9.2.17 Quantification and Statistical Analysis

Statistical analysis was performed using Prism 6 (GraphPad Software). Statistical significance was evaluated by Student's t test or one-way ANOVA. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant. Mean (±SEM) was indicated with horizontal lines. N represents number of biological replicates unless otherwise stated. Statistical details for each experiment can be found in its corresponding figure legends.

9.3 Data and Software Availability

The RNA-seq data is available in the ArrayExpress under the accession code E-MTAB-6028.

9.4 Table for Supplementary Materials

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti mouse I-A/I-E clone M5/114.15.2, Alexa Fluor 700	Biolegend	Cat#107622; RRID:AB_493727
Anti mouse CD11b clone M1/70, Brilliant Violet 650	Biolegend	Cat#101259; RRID:AB_2566568
Anti mouse CD11b clone M1/70, Percp-Cy5.5	Biolegend	Cat#101228; RRID:AB_893232
Anti mouse CD11c clone N418, PE-Cy7	Biolegend	Cat#117318; RRID:AB_493568
Anti mouse CD45 clone 30-F11, APC-Cy7	Biolegend	Cat#103116; RRID:AB_312981
Anti mouse CD45 clone 30-F11, Pacific Blue	Biolegend	Cat#103126; RRID:AB_493535
Anti mouse CD45.1 clone A20, Pacific Blue	Biolegend	Cat#110722; RRID:AB_492866
Anti mouse CD45.1 clone A20, APC	Biolegend	Cat#110714; RRID:AB_313503
Anti mouse CD45.2 clone 104, APC-Cy7	Biolegend	Cat#109824; RRID:AB_830789
Anti mouse CD45.2 clone 104, PB	Biolegend	Cat#109820; RRID:AB_492872
Anti mouse CD115 clone AFS98, APC	eBioscience	Cat#17115282; RRID:AB_1210789
Anti mouse Ly6C, clone AL-21, FITC	BD	Cat#553104; RRID:AB_394628

Anti mouse Ly6C HK1.4, Brilliant Violet 605	Biolegend	Cat#128036; RRID:AB_2562353
Anti mouse Ly6C HK1.4, Brilliant Violet 711	Biolegend	Cat#128037; RRID:AB_2562630
Anti mouse Ly6G clone 1A8, Percp-Cy5.5	Biolegend	Cat#127616; RRID:AB_1877271
Anti mouse Ly6G clone 1A8, Brilliant Violet 421	Biolegend	Cat#127628; RRID:AB_2562567
Anti mouse Ly6G clone 1A8, PE	Biolegend	Cat#127605; RRID:AB_1236488
Anti mouse Siglec-F clone E50-2440, PE-CF594	BD	Cat#562757; RRID:AB_2687994
Anti mouse CD3 clone 17A2, Alexa Fluor 700	eBioscience	Cat# 56-0032-82; RRID:AB_529507
Anti mouse CD45R clone RA3-6B2, PE-Cy7	Biolegend	Cat#103222; RRID:AB_313005
Anti mouse CD103 clone 2E7, APC	eBioscience	Cat# 17-1031-82 RRID:AB_1106992
Anti mouse F4/80 clone Cl:A3-1, Alexa Fluor 647	AbD Serotec	Cat# MCA497A647; AB_1102555
Anti mouse F4/80 clone Cl:A3-1, Biotin	AbD Serotec	Cat# MCA497B(B); RRID:AB_323893
Anti mouse CD64 clone X54-5/7.1, PE	Biolegend	Cat#139304; RRID: AB_10612740
Anti mouse CD24 clone M1/69, Percp-Cy5.5	Biolegend	Cat#101824; RRID:AB_1595491
Anti mouse CD24 clone M1/69, Alexa Fluor 488	Biolegend	Cat#101816; RRID:AB_493482
Anti mouse CD31 clone 390, FITC	Biolegend	Cat# 102406; RRID:AB_312901
Anti mouse EpCam clone G8.8, Percp-Cy5.5	Biolegend	Cat#118220; RRID:AB_2246499
Anti mouse FABP4 Biotinylated Antibody	R&D	Cat# BAF1443
Anti mouse CD16/CD32 clone 2.4G2	Biolegend	Cat#101310; RRID:AB_2103871
mouse IgG1 isotype control clone: MOPC-21	BioXcell	Cat# BE0083; RRID:AB_1107784
anti-pan TGF-β1 antibody clone: 1D11.16.8	BioXcell	Cat# BE0057; RRID:AB_1107757
Chemicals, Peptides, and Recombinant Proteins		
Collagenase type IV	Sigma-Aldrich	Cat#C5138-1G
Percoll	GE	Cat#P4937
HBSS (with Ca^{2+}/Mg^{2+})	Gibco	Cat#14025-050
HBSS (without Ca^{2+}/Mg^{2+}),	Gibco	Cat#14170-112
Dispase	Gibco	Cat#17105-041
Oil Red O (Sudan IV)	Sigma	Cat#O0625
Immunoselect antifading mounting medium	Dianova	Cat# SCR-38447
DPX mounting medium	Sigma	Cat#06522
Hematoxylin	Morphisto	Cat#10231.01000
Eosin	Morphisto	Cat#10177.00500
DAPI containing immunoselect antifading mounting medium	Dianova	Cat#SCR-038448
immunoselect antifading mounting medium	Dianova	Cat#SCR-038447
hTGF-β1	PeproTech	Cat#100-21
GM-CSF	Biolegend	Cat#576308

DMEM	PAN Biotech	Cat#P04-03500
Sodium Pyruvate	Gibco	Cat#11360
GlutaMAX	Gibco	Cat#35050-038
FCS	Merck	Cat#S0615
Pen/Strep	Gibco	Cat#15140-038
β-Mercaptoethanol	Gibco	Cat#31350-010
HEPES	Gibco	Cat#15630-056
Proteinase inhibitors	Roche	Cat#05056489001
Rosiglitazone	Sigma	Cat#R2408-50MG
Critical Commercial Assays		
Zombie Aqua Fixable Viability Kit	Biolegend	Cat# 423102
Zombie Near-IR Fixable Viability kit	Biolegend	Cat# 423106
RNeasy micro plus kit	Qiagen	Cat# 74034
M-MLV reverse transcriptase	Invitrogen	Cat# 28025
SYBR Green	Bio-Rad	Cat# 1725124
BCA protein assay kit	Pierce	Cat# 23227
mouse TGF-beta1 Elisa	R&D	Cat# DY1679-05
ELISA for SP-D	R&D	Cat# DY6839-05
TruSeq Stranded mRNA Sample Prep Kit	Illumina	Cat# RS-122
Deposited Data		
RNA-seq data		E-MTAB-6028
Experimental Models: Organisms/Strains		
Itgax^{Cre}	The Jackson Laboratory	RRID:IMSR_JAX:008068
Lyz2^{Cre}	The Jackson Laboratory	RRID:IMSR_JAX:004781
Vav1^{Cre}	Group Manfred Kopf	RRID:IMSR_JAX:008610
R26^{CreER}	The Jackson Laboratory	RRID:IMSR_JAX:004847
Cx3cr1^{CreER}	Group Steffen Jung	RRID:IMSR_JAX:021160
Tgfbr2$^{fl/fl}$	The Jackson Laboratory	RRID:IMSR_JAX:012603
Tgfb1$^{fl/fl}$	The Jackson Laboratory	RRID:IMSR_JAX:010721
Csf2rb$^{-/-}$ (*Csf2rb*$^{LacZ/LacZ}$)	Croxford et al., 2015	Croxford et al., 2015
Influenza virus (A/Puerto rico/8/1934)	Charles River	
Software and Algorithms		
GraphPad Prism 6	GraphPad Software	N/A
FlowJo v10	Tree Star	https://www.flowjo.com/solutions/flowjo/downloads
Cytobank	Cytobank	http://fluidigm.cytobank.org
Other		
22 G x 1.00 Insyte-W™ peripheral venous catheter	BD	Cat# 381323

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PUBLICATIONS

- **X. Yu**, A. Buttgereit, I. Lelios, S. G. Utz, D. Cansever, B. Becher, M. Greter, *The cytokine TGF-β promotes the development and homeostasis of alveolar macrophages.* ***Immunity****, 47, 903-912(2017)*
- A. Buttgereit, I. Lelios, **X. Yu**, M. Vrohlings, N.R. Krakoski, E.L. Gautier, R. Nishinakamura, B. Becher and M. Greter, *Sall1 is a transcriptional regulator defining microglia identity and function.* ***Nature Immunology****, 17, 1397–1406 (2016).*

RESEARCH SKILLS AND EXPERTISE

- Myeloid Cells, Mouse and Embryo Handling, Murine Models for Pulmonary and Systematic infection, Pulmonary Biology
- Flow Cytometry Analysis and Cell Sorting, Multidimensional Data Analysis, R-based Immune Cell Profiling
- RNAseq and Data Display
- Primary Cell Culture and Stimulation, Cell Culture, Lentivirus Production and Infection.
- qPCR, Elisa, Elispot, Western Blot, IP, ChIP, Molecular Cloning
- Fluorescent Microscopy, Live cell imaging, FRAP

HONORS

- MIM Travel Grant supported by the Microbiology and Immunology PhD program — 09.2016
- HBIGS Scholarship for Master Student, awarded by the Hartmut Hoffmann-Berling International Graduate School, Germany — 09.2011-02.2013